STIC-ILL

Adams

From: Gabel, Gailene 1641
Sent: Wednesday, November 08, 2000 8:38 AM
To: STIC-ILL

12-

Please provide a copy of the following:

1) Paz A et al., Phenotyping analysis of peripheral blood leukocytes in patients with multiple sclerosis
EUROPEAN JOURNAL OF NEUROLOGY, (MAY 1999) Vol. 6, No. 3, pp. 347-352.

2) Gabriel H.H.W. et al., Overtraining and immune system: A prospective longitudinal study in endurance athletes.
Medicine and Science in Sports and Exercise, (1998) 30/7 (1151-1157).

3) Loppow D.et al., Flow cytometric analysis of the effect of dithiothreitol on leukocyted surface markers.
European Respiratory Journal, (2000) 16/2 (324-329).

4) Han K et al., Human basophils express CD22 without expression of CD19
CYTOMETRY, (1999 Nov 1) 37 (3) 178-83.

5) Ohtsu S et al, Enhanced neutrophilic granulopoiesis in rheumatoid arthritis. Involvement of neutrophils in disease progression.
JOURNAL OF RHEUMATOLOGY, (2000 Jun) 27 (6) 1341-51.

6) Girodon F. et al., Immunophenotype of a transient myeloproliferative disorder in a newborn with trisomy 21.
Communications in Clinical Cytometry, (15 Apr 2000) 42/2 (118-122).

7) Hashimi L. et al., Cytofluorometric detection of chronic myelocytic leukemia supervening in a patient with chronic lymphocytic leukemia.
American Journal of Medicine, (1986) 80/2 (269-275).

8) Aardal N P et al., Sequential flowcytometric analysis of cellular DNA-content in peripheral blood during treatment for acute leukaemia.
SCANDINAVIAN JOURNAL OF HAEMATOLOGY, (1979 Jan) 22 (1) 25-32.

Thank you

Gail Gabel
305-0807
7B15
ASN 09/388,899

Gabel, Gailene

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JOURNAL OF RHEUMATOLOGY, (2000 Jun) 27 (6) 1341-51.

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SCANDINAVIAN JOURNAL OF HAEMATOLOGY, (1979 Jan) 22 (1) 25-32.

Thank you

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DB Name	Query	Hit Count	Set Name
USPT,JPAB,EPAB,DWPI,TDBD	cd45 and (cd16 and cd11b and cd66b and cd66c)	0	L24
USPT,JPAB,EPAB,DWPI,TDBD	l5 and (immature adj3 (granulocyt$ or neutrophil$))	2	L23
USPT,JPAB,EPAB,DWPI,TDBD	l20 and (neutrophil$ or granulocyt$)	63	L22
USPT,JPAB,EPAB,DWPI,TDBD	l20 and l12	2	L21
USPT,JPAB,EPAB,DWPI,TDBD	l19 and (flow cytomet$)	75	L20
USPT,JPAB,EPAB,DWPI,TDBD	l5 and leu?ocyt$	111	L19
USPT,JPAB,EPAB,TDBD,DWPI	('6043348' '5785869' '5776709' '5672346' '5958776' '5928949' '5538893' '5464752' '5260192' '5731206')[ABPN1,PN,TBAN,WKU]	26	L18
USPT,JPAB,EPAB,DWPI,TDBD	l13 and (antibod$)	17	L17
USPT,JPAB,EPAB,DWPI,TDBD	l13 and (immature)	19	L16
USPT,JPAB,EPAB,DWPI,TDBD	l12 and l5	2	L15
USPT,JPAB,EPAB,DWPI,TDBD	l13 and l5	2	L14
USPT,JPAB,EPAB,DWPI,TDBD	l12 and l2	58	L13
USPT,JPAB,EPAB,DWPI,TDBD	(leu?ocyt$) adj3 (classif$)	129	L12
USPT,JPAB,EPAB,DWPI,TDBD	l10 and l6	71	L11
USPT,JPAB,EPAB,DWPI,TDBD	l2 and l5	80	L10
USPT,JPAB,EPAB,DWPI,TDBD	l4 and l5	3	L9
USPT,JPAB,EPAB,DWPI,TDBD	l4 and l7	2	L8
USPT,JPAB,EPAB,DWPI,TDBD	l5 and l6	95	L7
USPT,JPAB,EPAB,DWPI,TDBD	fitc or pe or pe-cy5 or percp	43067	L6
USPT,JPAB,EPAB,DWPI,TDBD	cd45 and (cd16 or cd11b or cd66b or cd66c)	130	L5
USPT,JPAB,EPAB,DWPI,TDBD	l2 and l3	60	L4
USPT,JPAB,EPAB,DWPI,TDBD	(leu?cyt$ or (white blood)) adj3 differential	163	L3
USPT,JPAB,EPAB,DWPI,TDBD	(flow cytomet$) and (leu?ocyt$ or(white blood))	2047	L2
DWPI,USPT,EPAB,JPAB,TDBD	(flow cytomt$) and (leucocyt$ or (white blood))	0	L1

FILE 'EMBASE, SCISEARCH, BIOSIS, MEDLINE' ENTERED AT 07:36:58 ON 08 NOV 2000

L1	15093	S FLOW CYTOMET? AND (LEU!OCYT? OR (WHITE BLOOD))
L2	1023	S CD45 AND (CD11B OR CD16 OR CD66B OR CD66C)
L3	223	S L1 AND L2
L4	3722	S L1 AND (DIFFERENTIAL? OR CLASSIF? OR SUBSET?)
L5	107	S L4 AND L2
L6	49	S L5 AND (IMMATURE OR ?MYELOCYT? OR NEUTROPHIL? OR GRANULOCYT?
L7	22	DUP REM L6 (27 DUPLICATES REMOVED)
L8	67424	S ((WHITE BLOOD) OR LEU!OCYT?) (2A) (COUNT OR DIFFERENTIAL)
L9	2163	S L8 AND L1
L10	25	S L9 AND L2
L11	20	DUP REM L10 (5 DUPLICATES REMOVED)
L12	1578	S L8 AND ((IMMATURE (2A) (NEUTROPHIL? OR GRANULOCYT?)) OR (?MY
L13	67	S L12 AND L1
L14	1	S L13 AND L2
L15	2	S L13 AND (ANTIBOD? AND CD)
L16	33	S L12 AND L4
L17	23	DUP REM L16 (10 DUPLICATES REMOVED)
L18	33	S L13 AND ANTI?
L19	26	DUP REM L18 (7 DUPLICATES REMOVED)

L7 ANSWER 2 OF 22 SCISEARCH COPYRIGHT 2000 ISI (R)
ACCESSION NUMBER: 1999:374099 SCISEARCH
THE GENUINE ARTICLE: 194CN
TITLE: Phenotyping analysis of peripheral blood **leukocytes** in patients with multiple sclerosis
AUTHOR: Paz A; Fiszer U; Zaborski J; Korlak J; Czlonkowski A; Czlonkowska A (Reprint)
CORPORATE SOURCE: MED UNIV WARSAW, DEPT EXPT & CLIN PHARMACOL, KRAKOWSKIE PRZEDMIESCIE 26-28, PL-00927 WARSAW, POLAND (Reprint); MED UNIV WARSAW, DEPT EXPT & CLIN PHARMACOL, PL-00927 WARSAW, POLAND; INST PSYCHIAT & NEUROL, DEPT NEUROL 2, PL-02957 WARSAW, POLAND
COUNTRY OF AUTHOR: POLAND
SOURCE: EUROPEAN JOURNAL OF NEUROLOGY, (MAY 1999) Vol. 6, No. 3, pp. 347-352.
Publisher: LIPPINCOTT WILLIAMS & WILKINS, 227 EAST WASHINGTON SQ, PHILADELPHIA, PA 19106.
ISSN: 1351-5101.
DOCUMENT TYPE: Article; Journal
FILE SEGMENT: CLIN
LANGUAGE: English
REFERENCE COUNT: 35

ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB Multiple sclerosis (MS) is a central nervous disease thought to be elicited by an autoimmune process, Many studies in recent years have concentrated on finding the alterations in the peripheral blood immune profile in MS patients that would reflect disease activity. In the present study, we investigated surface antigen expression on lymphocytes and **granulocytes** from MS patients and control subjects. We have studied 29 patients suffering from relapsing-remitting or relapsing-progressive forms of MS. The disease was diagnosed in all patients at least 12 months before inclusion into the study. All patients had no attack at the study entry date or within a previous month. The control group included 29 age-matched subjects. Phenotyping of peripheral blood **leukocytes** was carried out with different fluorescence-conjugated murine monoclonal antibodies. The analysis was performed with three-color **flow cytometry**. The following antigens were determined [cluster of definition (CD)]: **leukocyte** common antigen (LCA) (B220, T 200, Ly-5), **CD45** ; LPS-R (lipopolysaccharide receptor), CD14; found on all T cells, CD3; LFA-2 (lymphocyte function associated antigen, T 11), CD2; coreceptor for MHC class II molecules, found on helper T cells, CD4; coreceptor for MHC class I molecules, found on suppressor/cytotoxic T cells, CD8; B4, found on all human B cells, CD19; NCAM (neural cell adhesion molecule), CD56; integrin beta 2 subunit, associated with CD11a (CD11a/CD18, LFA-1, alpha L beta 2) and **CD11b** (**CD11b**/CD18, Mac-1, CR3, alpha M beta 2), CD18; alpha L, alpha subunit of integrin LFA-1 (alpha L beta 2, CD11a/CD18), CD11a; alpha M, alpha subunit of integrin Mac-1 (CR3, alpha M beta 2, **CD11b**/CD18), **CD11b**; ICAM-1 (intercellular adhesion molecule), CD54; H-CAM, Hermes antigen, Pgp-1, CD44; AIM (activation inducer molecule), early activation antigen, CD69; T-cell receptor gamma delta, TCR gamma delta. In the MS group, we have found a significant increased expression of CD54 and CD44 antigens on lymphocytes, and higher percentage CD54(+) and CD11a(+)CD54(+) lymphocytes out of all

lymphocytes compared with the control group. We have also found a significant increased expression of CD11a, CD18 and CD54 antigens on **granulocytes**, and higher percentage **CD11b**(+)CD18(+) **granulocytes** out of all **granulocytes** in MS patients compared with control. Higher levels of expression of the adhesion molecules may reflect the activation state of **leukocytes** in MS patients. Eur J Neurol 6:347-352 (C) 1999 Lippincott Williams & Wilkins.

TI Phenotyping analysis of peripheral blood **leukocytes** in patients with multiple sclerosis

AB . . . in MS patients that would reflect disease activity. In the present study, we investigated surface antigen expression on lymphocytes and **granulocytes** from MS patients and control subjects. We have studied 29 patients suffering from relapsing-remitting or relapsing-progressive forms of MS. The. . . the study entry date or within a previous month. The control group included 29 age-matched subjects. Phenotyping of peripheral blood **leukocytes** was carried out with different fluorescence-conjugated murine monoclonal antibodies. The analysis was performed with three-color **flow cytometry**. The following antigens were determined [cluster of definition (CD)]: **leukocyte** common antigen (LCA) (B220, T 200, Ly-5), **CD45**; LPS-R (lipopolysaccharide receptor), CD14; found on all T cells, CD3; LFA-2 (lymphocyte function associated antigen, T 11), CD2; coreceptor for. . . NCAM (neural cell adhesion molecule), CD56; integrin beta 2 subunit, associated with CD11a (CD11a/CD18, LFA-1, alpha L beta 2) and **CD11b** (**CD11b**/CD18, Mac-1, CR3, alpha M beta 2), CD18; alpha L, alpha subunit of integrin LFA-1 (alpha L beta 2, CD11a/CD18), CD11a; alpha M, alpha subunit of integrin Mac-1 (CR3, alpha M beta 2, **CD11b**/CD18), **CD11b**; ICAM-1 (intercellular adhesion molecule), CD54; H-CAM, Hermes antigen, Pgp-1, CD44; AIM (activation inducer molecule), early activation antigen, CD69; T-cell receptor. . . compared with the control group. We have also found a significant increased expression of CD11a, CD18 and CD54 antigens on **granulocytes**, and higher percentage **CD11b**(+)CD18(+) **granulocytes** out of all **granulocytes** in MS patients compared with control. Higher levels of expression of the adhesion molecules may reflect the activation state of **leukocytes** in MS patients. Eur J Neurol 6:347-352 (C) 1999 Lippincott Williams & Wilkins.

ST Author Keywords: multiple sclerosis; peripheral blood lymphocytes; peripheral blood **granulocytes**; adhesion molecules

STP KeyWords Plus (R): T-CELL **SUBSETS**; CENTRAL-NERVOUS-SYSTEM; ADHESION MOLECULES; MONOCLONAL-ANTIBODIES; CEREBROSPINAL-FLUID; LYMPHOCYTES; PATHOGENESIS; MECHANISMS; DISABILITY; SERIAL

L7 ANSWER 4 OF 22 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.DUPLICATE 2

ACCESSION NUMBER: 1998231059 EMBASE

TITLE: Overtraining and immune system: A prospective longitudinal study in endurance athletes.

AUTHOR: Gabriel H.H.W.; Urhausen A.; Valet G.; Heidelbach U.; Kindermann W.

CORPORATE SOURCE: Dr. H.H.W. Gabriel, Inst. fur Sport-/Praventivmedizin, Universitat des Saarlandes, D-66041 Saarbrucken, Germany. gabriel@rz.uni-sb.de

SOURCE: Medicine and Science in Sports and Exercise, (1998) 30/7 (1151-1157).
Refs: 46
ISSN: 0195-9131 CODEN: MSCSBJ

COUNTRY: United States

DOCUMENT TYPE: Journal; Conference Article

FILE SEGMENT: 026 Immunology, Serology and Transplantation
035 Occupational Health and Industrial Medicine

LANGUAGE: English

SUMMARY LANGUAGE: English

AB A prospective longitudinal study investigated for 19 .+-. 3 months whether

immunophenotypes of peripheral **leukocytes** were altered in periods of severe training. **Leukocyte** membrane antigens (CD3, CD4, CD8, CD14, **CD16**, CD19, **CD45**, CD45RO, and CD56) of endurance athletes were immunophenotyped (dual-color **flow cytometry**) and list mode data analyzed by a self-learning **classification** system in a state of an overtraining syndrome (OT; N = 15) and several occasions without symptoms of staleness (NS; N = 70). Neither at physical rest nor after a short-term highly intensive cycle ergometer exercise session at 110% of the individual anaerobic threshold did cell counts of **neutrophils**, T, B, and natural killer cells differ between OT and NS. Eosinophils were lower during OT, activated T cells (CD3+HLA-DR+) showed slight increases (NS: 5.5 .+-. 2.7; OT 7.3 .+-. 2.4% CD3+ of cells; means .+-. SD; P <0.01) during OT without reaching pathological ranges. The cell-surface expression of CD45RO (P < 0.001) on T cells, but not cell concentrations of CD45RO+ T cells, were higher during OT. OT could be **classified** with high specificides (92%) and sensitivities (93%). It is concluded that OT does not lead to clinically relevant alterations of immunophenotypes in peripheral blood and especially that an immunosuppressive effect cannot be detected. Immunophenotyping may provide help with the diagnosis of OT in future, but the diagnostic approach presented here requires improvements before use in sports medical practice is enabled.

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CT Medical Descriptors:
*overexertion: DI, diagnosis
*overexertion: ET, etiology
*immune system
athlete
endurance
immunophenotyping
neutrophil
t lymphocyte
b lymphocyte
natural killer cell
flow cytometry
exercise
human
normal human
human cell
conference paper
leukocyte antigen
cd3 antigen
cd4 antigen
cd8 antigen
cd14 antigen
cd16 antigen
cd19 antigen

cd45 antigen
cd56 antigen

L11 ANSWER 1 OF 20 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.

ACCESSION NUMBER: 2000285656 EMBASE

TITLE: **Flow cytometric** analysis of the effect of dithiothreitol on **leukocyted** surface markers.

AUTHOR: Loppow D.; Bottcher M.; Gercken G.; Magnussen H.; Jorres R.A.

CORPORATE SOURCE: D. Loppow, Krankenhaus Grosshansdorf, Zent. fur Pneum. und Thoraxchirurgie, D-22927 Grosshansdorf, Germany

SOURCE: European Respiratory Journal, (2000) 16/2 (324-329). Refs: 26
ISSN: 0903-1936 CODEN: ERJOEI

COUNTRY: Denmark

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 005 General Pathology and Pathological Anatomy
015 Chest Diseases, Thoracic Surgery and Tuberculosis
037 Drug Literature Index

LANGUAGE: English

SUMMARY LANGUAGE: English

AB Pretreatmefit with dithiothreitol (DTT) is necessary to dissolve mucus in samples of induced sputum prior to analysis. However, DTT may affect cell surface markers which are essential for lymphocyte subtyping. Therefore, the aim of this study was to evaluate the effect of DTT on an appropriate panel of surface markers. Peripheral blood **leukocytes** were used because these cells, in contrast to sputum cells, could be obtained without DTT treatment. Peripheral blood from healthy donors was incubated with either DTT according to standard sputum procedures or phosphate-buffered saline (PBS), washed and incubated with fluorochrome-labelled antibodies. After lysis of erythrocytes, analysis was performed using a calibrated **flow cytometer**.
Leukocyte populations were identified by their light scattering properties. For analysis, fluorescence intensity was compared between DTT-
and PBS-treated samples. After treatment with DTT, fluorescence intensity was significantly increased in **CD16**-positive granulocytes; it was reduced in CD2-positive lymphocytes, **CD45**-positive lymphocytes and CD14-positive monocytes (p .ltoreq. 0.001). These changes occurred in all samples. The fluorescence intensity of CD3-, CD4-, CD8-, CD19-, CD56- and histocompatibility **leukocyte** antigen DR-positive lymphocytes was not altered by DTT. However, there were statistically significant ($p<0.001$), although small, changes in the percentages of **leukocytes**. The present data demonstrate that, although dithiothreitol as used in sputum analysis affects some surface markers of peripheral blood **leukocytes**, comparability between samples concerning lymphocyte surface markers is preserved. Therefore, it is suggested that treatment of sputum samples with dithiothreitol does not
invalidate the immunocytochemical analysis of lymphocytes.

TI **Flow cytometric** analysis of the effect of dithiothreitol on **leukocyted** surface markers.

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CT Medical Descriptors:
*sputum cytodiagnosis
*respiratory tract disease: DI, diagnosis
flow cytometry
leukocyte count
immunohistochemistry
laboratory test
diagnostic accuracy
human
male
female
human experiment
adult
article
priority journal
*dithiothreitol
leukocyte antigen: EC, endogenous compound
lymphocyte surface marker: EC, endogenous compound

L11 ANSWER 2 OF 20 MEDLINE
ACCESSION NUMBER: 1999451074 MEDLINE
DOCUMENT NUMBER: 99451074
TITLE: Human basophils express CD22 without expression of CD19 [see comments].
COMMENT: Comment in: Cytometry 2000 Jul 1;40(3):251
AUTHOR: Han K; Kim Y; Lee J; Lim J; Lee K Y; Kang C S; Kim W I; Kim B K; Shim S I; Kim S M
CORPORATE SOURCE: Department of Clinical Pathology, Catholic University Medical College, Seoul, Korea.. hankja@cmc.cuk.ac.kr
SOURCE: CYTOMETRY, (1999 Nov 1) 37 (3) 178-83.
Journal code: D92. ISSN: 0196-4763.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200001

AB BACKGROUND:Even modern automatic cell counters cannot count basophils precisely. Therefore, we need a rapid, accurate, precise, and easy method for counting basophils. METHODS:Using **flow cytometry**, basophils (CD22+/CD19-) and B cells (CD22+/CD19+) were counted. Within a large lymphocyte light scatter gate, % basophils (G%baso) and % B cells (G%B) were determined from the total count. Another method of analysis was to make two regions (R1 for basophils and R2 for B cells) and to determine in those the % basophils (R1%baso) and % B cells (R2%B) without gating. The **flow cytometric** basophil counts of the blood of 21 normal controls and 43 chronic myelogenous leukemia (CML) patients were compared with manual basophil count (Ma%baso) and basophil count by Coulter electronic cell counter (Hialeah, FL) (Auto%baso). CD22+/CD19- cells were sorted by a FACSCalibur (Becton Dickinson, San Jose, CA).

RESULTS:The G%baso of all samples was 4.66 +/- 5.35%, and R1%baso was 4.23
+/- 4.88%, and they were well-correlated (r = 0.996, P < 0.001). The G%B of all samples was 1.55 +/- 1.68%, and R2%B was 1.59 +/- 1.67%, and they were also well-correlated (r = 0.993, P < 0.001). Their correlation was better in normal controls than in CML. G%baso was well-correlated to Ma%baso (r = 0.827) and Auto%baso (r = 0.806), and R1%baso was well-correlated to Ma%baso (r = 0.831) but showed poor correlation to Auto%baso (r = 0.734). Auto%baso revealed the poorest correlation to Ma%baso (r = 0.692). The sorted CD22+/CD19- cells were all basophils (99.48 +/- 0.30%), and they revealed CD13, CD33, and dim **CD45** expression, whereas CD3, CD14, **CD16**, and HLA-DR were not detected on them. CONCLUSIONS: We discovered a specific marker combination
to identify basophils (CD22+/CD19-), and we suggest that **flow cytometric** analysis using these markers is an easy, reliable, and accurate method of basophil counting. Copyright 1999 Wiley-Liss, Inc.

AB . . . cell counters cannot count basophils precisely. Therefore, we need a rapid, accurate, precise, and easy method for counting basophils. METHODS:Using **flow cytometry**, basophils (CD22+/CD19-) and B cells (CD22+/CD19+) were counted. Within a large lymphocyte light scatter gate, % basophils (G%baso) and %. . . for B cells) and to determine in those the % basophils (R1%baso) and % B cells (R2%B) without gating. The **flow cytometric** basophil counts of the blood of 21 normal controls and 43 chronic myelogenous leukemia (CML) patients were compared with manual. . . (r = 0.692). The sorted CD22+/CD19- cells were all basophils (99.48 +/- 0.30%), and they revealed CD13, CD33, and dim **CD45** expression, whereas CD3, CD14, **CD16**, and HLA-DR were not detected on them. CONCLUSIONS: We discovered a specific marker combination to identify basophils (CD22+/CD19-), and we suggest that **flow cytometric** analysis using these markers is an easy, reliable, and accurate method of basophil counting. Copyright 1999 Wiley-Liss, Inc.

CT . . .

*Antigens, CD19: ME, metabolism
*Antigens, Differentiation, B-Lymphocyte: ME, metabolism
B-Lymphocytes: CY, cytology
Basophils: CY, cytology
*Basophils: ME, metabolism
Cell Separation
Flow Cytometry: MT, methods
Fluorescent Antibody Technique, Indirect
Immunophenotyping: MT, methods
Leukemia, Myeloid, Chronic: BL, blood
Leukemia, Myeloid, Chronic: ME, metabolism
Leukocyte Count: MT, methods

L11 ANSWER 10 OF 20 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.DUPLICATE 3
ACCESSION NUMBER: 97170006 EMBASE
DOCUMENT NUMBER: 1997170006
TITLE: Toward a new reference method for the **leukocyte** five-part **differential**.
AUTHOR: Hubl W.; Wolfbauer G.; Andert S.; Thum G.; Streicher J.; Hubner C.; Lapin A.; Bayer P.M.
CORPORATE SOURCE: W. Hubl, Central Lab, Wilhelminenspital, Montleartstrasse 37, A-1171 Vienna, Austria
SOURCE: Communications in Clinical Cytometry, (1997) 30/2 (72-84). Refs: 54
ISSN: 0196-4763 CODEN: CCCYEM
COUNTRY: United States
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 005 General Pathology and Pathological Anatomy
029 Clinical Biochemistry
LANGUAGE: English
SUMMARY LANGUAGE: English

AB A **flow cytometric** method performing a five-part **leukocyte differential** based on three-color staining with anti-**CD45**-fluorescein isothiocyanate (FITC), anti-CD-14-phycoerythrin (PE)/Cy5, and a cocktail of PE-labeled anti-CD2, anti-**CD16**, and anti-HLA-DR antibodies was evaluated. Results obtained by using three different sample preparation procedures and two different **flow cytometers** were compared with those of a 1,000-cell manual differential for evaluation of accuracy. We observed excellent correlations with the manual **differential** for all **leukocyte** subclasses and even higher correlations between the different **flow cytometric** methods. **Flow cytometric** basophil results were identical to the manual counts, regardless of which sample preparation technique or **flow cytometer** was used. Therefore, we propose our **flow cytometric** method as the first acceptable automated reference method for basophil counting. The **flow cytometric** results for the other **leukocyte** subclasses were apparently influenced by the sample preparation, which could not be explained by cell loss during washing steps. Moreover, a small influence of the **flow cytometer** was also observed. Assessing the influence of sample storage, we found only minimal changes within 24 h. In establishing reference values, high precision of **flow cytometric** results facilitated detection of a significantly higher monocyte count for males (relative count: 7.08 .+-. 1.73% vs. 6.44 .+-. 1.33% P < 0.05; absolute count: 0.530 .+-. 0.181 x 109/liter vs. 0.456 .+-. 139 x 109/liter, P < 0.01). Our data indicate that monoclonal antibody-based **flow cytometry** is a highly suitable reference method for the five-part differential: It also shows, however, that studies will have to put more emphasis on methodological issues to define a method that shows a high interlaboratory reproducibility.

TI Toward a new reference method for the **leukocyte** five-part **differential**.

AB A **flow cytometric** method performing a five-part **leukocyte differential** based on three-color staining with anti-**CD45**-fluorescein isothiocyanate (FITC), anti-CD-14-phycoerythrin (PE)/Cy5, and a cocktail of PE-labeled anti-CD2,

anti-**CD16**, and anti-HLA-DR antibodies was evaluated. Results obtained by using three different sample preparation procedures and two different **flow cytometers** were compared with those of a 1,000-cell manual differential for evaluation of accuracy. We observed excellent correlations with the manual **differential** for all **leukocyte** subclasses and even higher correlations between the different **flow cytometric** methods. **Flow cytometric** basophil results were identical to the manual counts, regardless of which sample preparation technique or **flow cytometer** was used. Therefore, we propose our **flow cytometric** method as the first acceptable automated reference method for basophil counting. The **flow cytometric** results for the other **leukocyte** subclasses were apparently influenced by the sample preparation, which could not be explained by cell loss during washing steps. Moreover, a small influence of the **flow cytometer** was also observed. Assessing the influence of sample storage, we found only minimal changes within 24 h. In establishing reference values, high precision of **flow cytometric** results facilitated detection of a significantly higher monocyte count for males (relative count: 7.08 .+-. 1.73% vs. 6.44 .+-. 1.33%. . . 0.530 .+-. 0.181 x 109/liter vs. 0.456 .+-. 139 x 109/liter, P < 0.01). Our data indicate that monoclonal antibody-based **flow cytometry** is a highly suitable reference method for the five-part differential: It also shows, however, that studies will have to put. . .

CT Medical Descriptors:

***leukocyte count**
article
automation
diagnostic accuracy
flow cytometry
human
human cell
priority journal
reproducibility

L14 ANSWER 1 OF 1 MEDLINE
ACCESSION NUMBER: 91255675 MEDLINE
DOCUMENT NUMBER: 91255675
TITLE: Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor **flow cytometry**.
AUTHOR: Bender J G; Unverzagt K L; Walker D E; Lee W; Van Epps D E;
Smith D H; Stewart C C; To L B
CORPORATE SOURCE: Applied Sciences, Baxter Healthcare Corporation, Round Lake, IL..
SOURCE: BLOOD, (1991 Jun 15) 77 (12) 2591-6.
Journal code: A8G. ISSN: 0006-4971.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals; Cancer Journals
ENTRY MONTH: 199109

AB Four-color **flow cytometry** was used with a cocktail of antibodies to identify and isolate CD34+ hematopoietic progenitors from normal human peripheral blood (PB) and bone marrow (BM). Mature cells that did not contain colony forming cells were resolved from immature cells using antibodies for T lymphocytes (CD3), B lymphocytes (CD20), monocytes (CD14), and **granulocytes** (**CD11b**). **Immature** cells were subdivided based on the expression of antigens found on hematopoietic progenitors (CD34, HLA-DR, CD33, CD19, **CD45**, CD71, CD10, and CD7). CD34+ cells were present in the circulation in about one-tenth the concentration of BM (0.2% v 1.8%) and had a different spectrum of antigen expression. A higher proportion of PB-CD34+ cells expressed the CD33 myeloid antigen (84% v 43%) and expressed higher levels of the pan **leukocyte** antigen **CD45** than BM-CD34+ cells. Only a small fraction of PB-CD34+ cells expressed CD71 (transferrin receptors) (17%) while 94% of BM-CD34+ expressed CD71+. The proportion of PB-CD34+ cells expressing the B-cell antigens CD19 (10%) and CD10 (3%) was not significantly different from BM-CD34+ cells (14% and 17%, respectively). Few CD34+ cells in BM (2.7%) or PB (7%) expressed the T-cell antigen CD7. CD34+ cells were found to be predominantly HLA-DR+, with a wide range of intensity. These studies show that CD34+ cells and their subsets can be identified in normal PB and that the relative frequency of these cells and their subpopulations differs in PB versus BM.

TI Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor **flow cytometry**.

AB Four-color **flow cytometry** was used with a cocktail of antibodies to identify and isolate CD34+ hematopoietic progenitors from normal human peripheral blood (PB). . . colony forming cells were resolved from immature cells using antibodies for T lymphocytes (CD3), B lymphocytes (CD20), monocytes (CD14), and **granulocytes** (**CD11b**). **Immature** cells were subdivided based on the expression of antigens found on hematopoietic progenitors (CD34, HLA-DR, CD33, CD19, **CD45**, CD71, CD10, and CD7). CD34+ cells were present in the circulation in about one-tenth the concentration of BM (0.2% v. . . higher proportion of PB-CD34+ cells expressed the CD33 myeloid antigen

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CT . . . Check Tags: Comparative Study; Human
Adult
Antibodies, Monoclonal
*Antigens, CD: AN, analysis
Antigens, Differentiation: AN, analysis
*Bone Marrow: CY, cytology
Flow Cytometry
*Hematopoietic Stem Cells: CY, cytology
Hematopoietic Stem Cells: IM, immunology
Histocompatibility Antigens: AN, analysis
HLA-DR Antigens: AN, analysis
Immunophenotyping
Leukocyte Count
Receptors, Transferrin: AN, analysis

CN 0 (Antibodies, Monoclonal); 0 (Antigens, CD); 0 (Antigens, CD34); 0 (Antigens, **CD45**); 0 (Antigens, Differentiation); 0 (Histocompatibility Antigens); 0 (HLA-DR Antigens); 0 (Receptors, Transferrin)

L19 ANSWER 1 OF 26 MEDLINE
ACCESSION NUMBER: 2000483495 MEDLINE
DOCUMENT NUMBER: 20309092
TITLE: Enhanced neutrophilic granulopoiesis in rheumatoid arthritis. Involvement of neutrophils in disease progression.
AUTHOR: Ohtsu S; Yagi H; Nakamura M; Ishii T; Kayaba S; Soga H; Gotoh T; Rikimaru A; Kokubun S; Itoh T
CORPORATE SOURCE: Department of Orthopedic Surgery, Tohoku University School of Medicine and Naruko National Hospital, Miyagi, Japan.
SOURCE: JOURNAL OF RHEUMATOLOGY, (2000 Jun) 27 (6) 1341-51. Journal code: JWX. ISSN: 0315-162X.
PUB. COUNTRY: Canada
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200012
ENTRY WEEK: 20001203
AB OBJECTIVE: To investigate enhanced granulopoiesis in bone marrow of patients with rheumatoid arthritis (RA), and the role of neutrophils in RA pathogenesis. METHODS: Aspirated bone marrow cells and peripheral blood **leukocytes** from patients with RA and non-RA patient controls were analyzed morphologically and by 2 color **flow cytometry** . Thirteen iliac bones (8 RA, 5 non-RA) were examined by light and transmission electron microscope (TEM). RESULTS: The percentage of CD15+CD16- cells (**immature neutrophils**) in RA bone marrow (64.3+/-13.4%, mean +/- SD) increased significantly compared to that of non-RA controls (43.2+/-14.3%), whereas the fraction of CD15+CD]6+ cells (mature neutrophils)was greatly decreased (RA 21.8+/-10.1%; non-RA 38.1+/-8.9%). The absolute number of CD15+CD16- cells also increased markedly in RA bone marrow. The ratio of immature cells to the total granulocytes (% CD15+CD16- to % CD15+) correlated with the Lansbury Index score (R = 0.76, p<0.0001). TEM observations revealed that abundant **immature neutrophils** adhered closely to the trabeculae of the iliac bone. Margins of trabeculae were mostly irregular, especially in severe RA, and collagenous fibers frequently disappeared in those trabeculae with ragged margins. CONCLUSION: In RA bone marrow, **immature neutrophils** (CD15+CD16-) were markedly increased in number; by contrast, no changes were found for mature cells. Augmented production of **immature neutrophils** (at the **promyelocyte**-to-**myelocyte** stage) might lead to the destruction of collagenous fibers in RA bone trabeculae, as revealed by TEM. Generalized bone destruction in RA might, at least in part, be caused by enhanced production of **immature neutrophils**.
AB . . . with rheumatoid arthritis (RA), and the role of neutrophils in RA pathogenesis. METHODS: Aspirated bone marrow cells and peripheral blood **leukocytes** from patients with RA and non-RA patient controls were analyzed morphologically and by 2 color **flow cytometry** . Thirteen iliac bones (8 RA, 5 non-RA) were examined by light and transmission electron microscope (TEM). RESULTS: The percentage of CD15+CD16- cells (**immature neutrophils**) in RA bone marrow (64.3+/-13.4%, mean +/- SD) increased significantly compared to that of non-RA controls (43.2+/-14.3%), whereas the fraction. . . (% CD15+CD16- to % CD15+) correlated with the Lansbury Index score (R = 0.76,

p<0.0001). TEM observations revealed that abundant **immature neutrophils** adhered closely to the trabeculae of the iliac bone. Margins of trabeculae were mostly irregular, especially in severe RA, and collagenous fibers frequently disappeared in those trabeculae with ragged margins. CONCLUSION: In RA bone marrow, **immature neutrophils** (CD15+CD16-) were markedly increased in number; by contrast, no changes were found for mature cells. Augmented production of **immature neutrophils** (at the **promyelocyte**-to-**myelocyte** stage) might lead to the destruction of collagenous fibers in RA bone trabeculae, as revealed by TEM. Generalized bone destruction in RA might, at least in part, be caused by enhanced production of **immature neutrophils**.

CT Check Tags: Female; Human; Male; Support, Non-U.S. Gov't
Adult
Aged
Aged, 80 and over
Antigens, CD15: AN, analysis
*Arthritis, Rheumatoid: IM, immunology
Bone Marrow Cells: CH, chemistry
Bone Marrow Cells: CY, cytology
Bone Marrow Cells: IM, immunology
Cell Aging: IM, immunology
Cell Division: IM, immunology
Disease Progression
Flow Cytometry
Ilium: IM, immunology
Ilium: UL, ultrastructure
Leukocyte Count
*Leukopoiesis: IM, immunology
Microscopy, Electron
Middle Age
Neutrophils: CH, chemistry
*Neutrophils: CY, cytology
*Neutrophils: IM, immunology
Receptors, IgG:. . .

CN 0 (**Antigens**, CD15); 0 (Receptors, IgG)

L19 ANSWER 2 OF 26 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.

ACCESSION NUMBER: 2000159349 EMBASE

TITLE: Immunophenotype of a transient myeloproliferative disorder in a newborn with trisomy 21.

AUTHOR: Girodon F.; Favre B.; Couillaud G.; Carli P.-M.; Parmeland C.; Maynadie M.

CORPORATE SOURCE: M. Maynadie, Hematology Laboratory, C.H.U. de Dijon, B.P. 1543, 21034 Dijon Cedex, France. mmaynadie@chu-dijon.fr

SOURCE: Communications in Clinical Cytometry, (15 Apr 2000) 42/2 (118-122).
Refs: 15
ISSN: 0196-4763 CODEN: CCCYEM

COUNTRY: United States

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 005 General Pathology and Pathological Anatomy
007 Pediatrics and Pediatric Surgery

LANGUAGE: English

SUMMARY LANGUAGE: English

AB Cytologic, immunologic, and cytogenetic studies were performed on the blast cells of a newborn with Down syndrome and transient myeloproliferative disease. This hematologic disorder is uncommon, and occurs primarily in infants with Down syndrome. This boy presented with a high **white blood** cell **count** and a high percentage of blast cells, without anemia or thrombocytopenia. Chromosome analysis showed a constitutional trisomy 21 without any other clonal abnormality. A three-color **flow cytometric** analysis was performed and revealed two different CD45 dim, CD34+, CD117+, CD56+ immature subpopulations: the normal immature myeloid precursor and an immature blast cell population that expressed CD41, CD42, CD61, CD36,

CD13, CD1a, and CD2. We postulate that this population could be the leukemic precursor involved in the acute megakaryoblastic leukemia frequently observed in children with Down syndrome. (C) 2000 Wiley-Liss, Inc.

AB . . . disease. This hematologic disorder is uncommon, and occurs primarily in infants with Down syndrome. This boy presented with a high **white blood** cell **count** and a high percentage of blast cells, without anemia or thrombocytopenia. Chromosome analysis showed a constitutional trisomy 21 without any other clonal abnormality. A three-color **flow cytometric** analysis was performed and revealed two different CD45 dim, CD34+, CD117+, CD56+ immature subpopulations: the normal immature myeloid precursor and. . .

CT Medical Descriptors:
*trisomy 21
*myeloproliferative disorder
immunophenotyping
blast cell
flow cytometry
promyelocyte
human
male
case report
newborn
article
priority journal
CD45 antigen: EC, endogenous compound
CD34 antigen: EC, endogenous compound
stem cell factor receptor: EC, endogenous compound
CD56 antigen: EC, endogenous compound
fibrinogen receptor: EC, endogenous compound
CD36 antigen: EC, endogenous compound
CD1 antigen: EC, endogenous compound
CD2 antigen: EC, endogenous compound

L19 ANSWER 23 OF 26 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.DUPLICATE 4
ACCESSION NUMBER: 86129835 EMBASE
DOCUMENT NUMBER: 1986129835
TITLE: Cytofluorometric detection of chronic **myelocytic** leukemia supervening in a patient with chronic lymphocytic leukemia.
AUTHOR: Hashimi L.; Al-Katib A.; Mertelsmann R.; et al.
CORPORATE SOURCE: Memorial Sloan-Kettering Cancer Center, New York, NY, United States
SOURCE: American Journal of Medicine, (1986) 80/2 (269-275). CODEN: AJMEAZ
COUNTRY: United States
DOCUMENT TYPE: Journal
FILE SEGMENT: 025 Hematology
016 Cancer
020 Gerontology and Geriatrics
006 Internal Medicine
005 General Pathology and Pathological Anatomy
LANGUAGE: English

AB An 82-year-old woman with stage I chronic lymphocytic leukemia presented with systemic symptoms, minimal adenopathy, hepatosplenomegaly, and anemia five years after the initial diagnosis was made and while receiving no therapy. Her **white blood** cell **count** was 231,000/mm3 with an absolute neutrophil count of 164,360/mm3 and lymphocyte count of 43,890/mm3. Peripheral blood smear inspection revealed both increased mature lymphocytes and myeloid cells at all stages of maturation. **Flow cytometric** analysis of forward- and right-angle light scatters demonstrated the presence of two populations of

cells, one lymphoid, bearing predominantly lambda light chain surface immunoglobulin and showing phenotypic characteristics of B cell chronic lymphocytic leukemia (HLA-DR-positive, BL-1-positive, and BL-2-positive, BL-7-positive, Leu-1-positive, Leu-10-positive, BL-5-negative, BL-6-negative, and OKM1-negative), and another granulocytic population expressing phenotypic features compatible with myeloid lineage (HLA-DR-negative, Leu-1-negative, BL-1-negative, BL-2-negative, BL-7-negative, Leu-10-negative, BL-5-positive, BL-6-negative, OKM1-positive, and surface immunoglobulin-negative). All of the peripheral blood cell metaphases were Philadelphia chromosome-positive after 24 hours of culture, confirming the diagnosis of chronic **myelocytic** leukemia, whereas all of the Epstein-Barr virus-treated B lymphocyte metaphases showed a normal karyotype after two weeks of culture. In this patient, analysis of surface **antigens** and immunoglobulin fractions by **flow cytometry** proved to be useful in recognizing concomitantly expressed leukemic lineages. This approach allows the increasing recognition of the heterogeneity of leukemic populations.

TI Cytofluorometric detection of chronic **myelocytic** leukemia supervening in a patient with chronic lymphocytic leukemia.

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Her **white blood** cell **count** was 231,000/mm3 with an absolute neutrophil count of 164,360/mm3 and lymphocyte count of 43,890/mm3. Peripheral blood smear inspection revealed both increased mature lymphocytes and myeloid cells at all stages of maturation. **Flow cytometric** analysis of forward- and right-angle light scatters demonstrated the presence of two populations of cells, one lymphoid, bearing predominantly lambda. . . All of the peripheral blood cell metaphases were Philadelphia chromosome-positive after 24 hours of culture, confirming the diagnosis of chronic **myelocytic** leukemia, whereas all of the Epstein-Barr virus-treated B lymphocyte metaphases showed a normal karyotype after two weeks of culture. In this patient, analysis of surface **antigens** and immunoglobulin fractions by **flow cytometry** proved to be useful in recognizing concomitantly expressed leukemic lineages. This approach allows the increasing recognition of the heterogeneity of. . .

L19 ANSWER 24 OF 26 MEDLINE

ACCESSION NUMBER: 86026767 MEDLINE
DOCUMENT NUMBER: 86026767
TITLE: Acute mixed lineage leukemia: clinicopathologic correlations and prognostic significance.
AUTHOR: Mirro J; Zipf T F; Pui C H; Kitchingman G; Williams D; Melvin S; Murphy S B; Stass S
CONTRACT NUMBER: RR0558414 (NCRR)
CA20180 (NCI)
CA21765 (NCI)
SOURCE: BLOOD, (1985 Nov) 66 (5) 1115-23.
Journal code: A8G. ISSN: 0006-4971.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals; Cancer Journals
ENTRY MONTH: 198602

AB The frequency and clinical significance of acute leukemia displaying both lymphoid and myeloid characteristics was determined in 123 consecutive children using a panel of lineage-associated markers. The leukemic blasts from 18 of 95 children (19%) with the diagnosis of acute lymphoblastic leukemia (ALL) by standard diagnostic criteria expressed myeloid-associated cell surface **antigens**. Despite immunological

evidence of lymphoid differentiation (17 CALLA + and one T cell-associated
antigen +) and findings of immunoglobulin gene rearrangement, blasts from these patients reacted with one to five monoclonal **antibodies** identifying myeloid-associated cell surface **antigens** (My-1, MCS.2, Mo1, SJ-D1, or 5F1). Dual staining with microsphere-conjugated **antibodies** and analysis by **flow cytometry** confirmed that some blasts were simultaneously expressing lymphoid- and myeloid-associated **antigens**. Conversely, blasts from seven of 28 patients (25%) with acute nonlymphocytic leukemia (ANLL), diagnosed by otherwise standard morphological and cytochemical criteria, expressed lymphoid-associated surface **antigens**. Dual staining of individual blasts demonstrated simultaneous expression of myeloperoxidase (MPO) (including Auer rods) in association with either T-11, CALLA, or terminal deoxynucleotidyl transferase. Blasts from one patient with ANLL demonstrated T cell receptor gene rearrangement, while blasts from another
patient demonstrated characteristics associated with T (T-11), B (CALLA and heavy-chain immunoglobulin gene rearrangement), and myeloid (MPO) lineage. There were no consistent cytogenetic abnormalities, and no patient demonstrated independent leukemic clones. Each patient with typical ALL, except for myeloid-associated **antigens**, achieved complete remission with conventional induction therapy for ALL. By contrast, three of the seven children with ANLL whose blasts expressed the
T-11 surface **antigen** failed ANLL induction therapy. These three patients subsequently achieved remission with ALL therapy.

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antigen +) and findings of immunoglobulin gene rearrangement, blasts from these patients reacted with one to five monoclonal **antibodies** identifying myeloid-associated cell surface **antigens** (My-1, MCS.2, Mo1, SJ-D1, or 5F1). Dual staining with microsphere-conjugated **antibodies** and analysis by **flow cytometry** confirmed that some blasts were simultaneously expressing lymphoid- and myeloid-associated **antigens**. Conversely, blasts from seven of 28 patients (25%) with acute nonlymphocytic leukemia (ANLL), diagnosed by otherwise standard morphological and cytochemical criteria, expressed lymphoid-associated surface **antigens**. Dual staining of individual blasts demonstrated simultaneous expression of myeloperoxidase (MPO) (including Auer rods) in association with either T-11, CALLA,. . . were no consistent cytogenetic abnormalities, and no patient demonstrated independent leukemic clones. Each patient with typical ALL, except for myeloid-associated **antigens**, achieved complete remission with conventional induction therapy for ALL. By contrast, three of the seven children with ANLL whose blasts expressed the T-11 surface **antigen** failed ANLL induction therapy. These three patients subsequently achieved remission with ALL therapy.

CT Check Tags: Female; Human; Male; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.
Acute Disease
Adolescence
Age Factors
Antibodies, Monoclonal: IM, immunology
Antigens, Neoplasm: AN, analysis
Antigens, Surface: AN, analysis
B-Lymphocytes: IM, immunology
Child
Child, Preschool
Genes, MHC Class II
Infant

*Leukemia: CL, classification
*Leukemia, Lymphocytic: IM, immunology
Leukemia, Myelocytic, Acute: IM, immunology
Leukocyte Count
Microspheres
Prognosis
*Tumor Stem Cells: CL, classification
CN 0 (**Antibodies**, Monoclonal); 0 (**Antigens**, Neoplasm); 0 (**Antigens**, Surface)

L19 ANSWER 26 OF 26 MEDLINE
ACCESSION NUMBER: 79138573 MEDLINE
DOCUMENT NUMBER: 79138573
TITLE: Sequential **flow cytometric** analysis of cellular DNA-content in peripheral blood during treatment for acute leukaemia.
AUTHOR: Aardal N P; Talstad I; Laerum O D
SOURCE: SCANDINAVIAN JOURNAL OF HAEMATOLOGY, (1979 Jan) 22 (1) 25-32.
Journal code: UCV. ISSN: 0036-553X.
PUB. COUNTRY: Denmark
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 197907
AB Sequential **flow cytometric** analysis (FCM) of relative nuclear DNA content per cell was done in peripheral blood of 12 patients during treatment for acute leukaemia. A marked increase of cells with S-phase DNA-content during the first hours of treatment was found in patients responding favorably to treatment. One patient with increase of 'S-phase cells' died before clinical improvement could be evaluated. However, lack of S-phase increase at one treatment cycle did not exclude a favorable response in the next. Two cases with probable aneuploid leukaemia showed gradual disappearance of abnormal cells during therapy. The value of FCM analysis of peripheral blood seems to be in predicting the response to treatment before clinical signs appear.
TI Sequential **flow cytometric** analysis of cellular DNA-content in peripheral blood during treatment for acute leukaemia.
AB Sequential **flow cytometric** analysis (FCM) of relative nuclear DNA content per cell was done in peripheral blood of 12 patients during treatment for. . .
CT Check Tags: Female; Human; Male
Adolescence
Adult
Aged
Antineoplastic Agents: TU, therapeutic use
Bone Marrow: CY, cytology
Cell Cycle
Cell Nucleus: AN, analysis
*Cytological Techniques
Drug Therapy, Combination
*DNA, Neoplasm: BL, blood
Leukemia, Lymphocytic: BL, blood
*Leukemia, Lymphocytic: DT, drug therapy
Leukemia, Myelocytic, Acute: BL, blood
***Leukemia, Myelocytic, Acute: DT, drug therapy**
Leukocyte Count
***Leukocytes: AN, analysis**
Middle Age
Spectrometry, Fluorescence

ND 3/9/11

STIC-ILL

From: Gabel, Gailene
Sent: Wednesday, November 08, 2000 8:38 AM
To: STIC-ILL

Please provide a copy of the following:

1) Paz A et al., Phenotyping analysis of peripheral blood leukocytes in patients with multiple sclerosis
EUROPEAN JOURNAL OF NEUROLOGY, (MAY 1999) Vol. 6, No. 3, pp. 347-352.

2) Gabriel H.H.W. et al., Overtraining and immune system: A prospective longitudinal study in endurance athletes.
Medicine and Science in Sports and Exercise, (1998) 30/7 (1151-1157).

3) Loppow D.et al., Flow cytometric analysis of the effect of dithiothreitol on leukocyted surface markers.
European Respiratory Journal, (2000) 16/2 (324-329).

12034858

4) Han K et al., Human basophils express CD22 without expression of CD19
CYTOMETRY, (1999 Nov 1) 37 (3) 178-83.

5) Ohtsu S et al, Enhanced neutrophilic granulopoiesis in rheumatoid arthritis. Involvement of neutrophils in disease progression.
JOURNAL OF RHEUMATOLOGY, (2000 Jun) 27 (6) 1341-51.

6) Girodon F. et al., Immunophenotype of a transient myeloproliferative disorder in a newborn with trisomy 21.
Communications in Clinical Cytometry, (15 Apr 2000) 42/2 (118-122).

20259369

7) Hashimi L. et al., Cytofluorometric detection of chronic myelocytic leukemia supervening in a patient with chronic lymphocytic leukemia.
American Journal of Medicine, (1986) 80/2 (269-275).

8) Aardal N P et al., Sequential flowcytometric analysis of cellular DNA-content in peripheral blood during treatment for acute leukaemia.
SCANDINAVIAN JOURNAL OF HAEMATOLOGY, (1979 Jan) 22 (1) 25-32.

Thank you

Gail Gabel
305-0807
7B15
ASN 09/388,899

Cytometry (Communications in Clinical Cytometry) 42:118–122 (2000)

Immunophenotype of a Transient Myeloproliferative Disorder in a Newborn With Trisomy 21

François Girodon,[1] Bernardine Favre,[1] Gérard Couillaud,[2] Paule-Marie Carli,[1] Chantal Parmeland,[1] and Marc Maynadié[1]*

[1]Hematology Laboratory, University Hospital, Dijon, France

[2]Department of Pediatric Oncology, University Hospital, Dijon, France

Cytologic, immunologic, and cytogenetic studies were performed on the blast cells of a newborn with Down syndrome and transient myeloproliferative disease. This hematologic disorder is uncommon, and occurs primarily in infants with Down syndrome. This boy presented with a high white blood cell count and a high percentage of blast cells, without anemia or thrombocytopenia. Chromosome analysis showed a constitutional trisomy 21 without any other clonal abnormality. A three-color flow cytometric analysis was performed and revealed two different CD45 dim, $CD34^+$, $CD117^+$, $CD56^+$ immature subpopulations: the normal immature myeloid precursor and an immature blast cell population that expressed CD41, CD42, CD61, CD36, CD13, CD1a, and CD2. We postulate that this population could be the leukemic precursor involved in the acute megakaryoblastic leukemia frequently observed in children with Down syndrome. Cytometry (Comm. Clin. Cytometry) 42:118–122, 2000. © 2000 Wiley-Liss, Inc.

Key terms: transient myeloproliferative disorder; immunophenotype; Down syndrome; megakaryocytic progenitors; myeloid progenitors

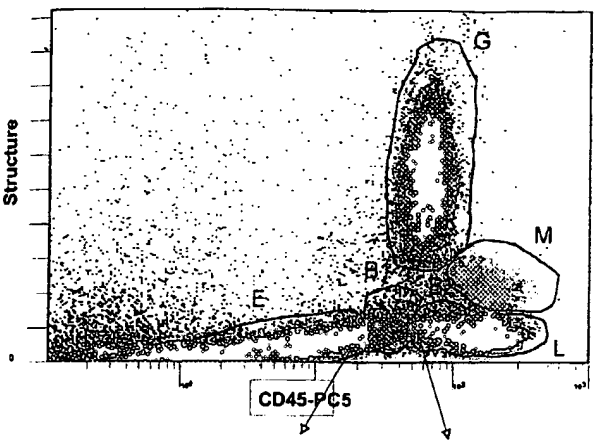
Transient myeloproliferative disorder (TMD) is known to be rare, and mainly affects children with Down syndrome. TMD is characterized by an increased number of white blood cells (WBC), often with a high percentage of blast cells, usually without anemia or thrombocytopenia (3). This situation is worrying and some parameters have been determined to distinguish it more easily from a true acute leukemia (3). In TMD, higher hemoglobin concentration and platelet count are noted, and the percentage of blast cells in bone marrow is often surprisingly lower than in peripheral blood (3,12). An isolated trisomy 21 is observed.

Limited data is available about immunophenotype of TMD blast cells because the majority of TMD cases reported were studied prior to the development of multicolor flow cytometric immunophenotyping techniques and to the description of new antigens (3,5,6,12). Here we report the phenotype of two blast-cell populations in a case of TMD studied by a three-color flow cytometric method.

CASE REPORT

A white boy was born in March 1999, at 35 weeks gestation. He is the third child of a 35-year-old mother, and presented with typical features of Down syndrome including a severe cardiac malformation. The initial WBC count was 75×10^9 cells/l with 57% blast cells, 39% segmented neutrophils, 3% lymphocytes, and 1% monocytes. Few micromegakaryoblasts, very large platelets, and 10% of erythroblasts were noted. The hemoglobin was 133 g/l and the platelet count was 398×10^9/l. Blast cells, with the May-Grünwald-Giemsa staining, appeared large, often with one or more nucleoli, without granules nor Auer rods into the cytoplasm. Myeloperoxydase and naphtyl butyrate esterase stainings were negative in all blast cells. In the bone marrow aspirate, similar blast cells were observed, but in lower proportions (23%). No morphologic abnormality was observed in other cell populations. Chromosome analysis performed on the bone marrow aspirate showed a trisomy 21 (47, XY, +21) without any other abnormality. The course of WBC and blast cell count showed a decrease over 2 weeks, and when the child was 16 days old, the WBC count was 10×10^9/l with 7% blast cells. The spontaneous regression of the blast cells lead us to consider this disorder as a TMD, and no chemotherapy was given. Now, 6 months later, the WBC count remains normal, the blast cells have disappeared and the child is healthy.

*Correspondence to: Marc Maynadié, Hematology Laboratory, C.H.U. de Dijon, B.P. 1543, 21034, Dijon Cedex, France.
E-mail: mmaynadie@chu-dijon.fr
Received 8 June 1999; Accepted 8 November 1999



	B1	B2
CD 34	93	66
CD 117	93	54
CD 38	9	63
CD 41	96	18
CD 42	89	18
CD 61	92	17
CD 36	87	44
CD 71	17	64
CD 15	3	2
Glyco A	2	12
CD 13	88	16
CD 33	14	64
CD 1a	86	18
CD 2	83	18
mCD 3	4	13
cCD 3	6	4
TCR αβ	1	2
TCR γδ	0	0
CD 5	1	9
CD 7	15	80
CD 10	0	0
CD 19	0	0
CD 56	90	71
CD 24	13	3

FIG. 1. CD45 expression on peripheral blood cells (DataMATE for Windows). Two CD45 dim populations were noted (B1 and B2), corresponding to the blast-cell subpopulations, with two different immunophenotypes. Other populations were erythroblasts (E), granular cells (G), lymphocytes (L), and monocytes (M).

MATERIALS AND METHODS

Immunophenotype was performed on whole blood, using a three-color flow cytometric method. In Brief, 10^6 WBC were added to a test tube with 10 µl of each directly conjugated monoclonal antibody. The cells were incubated in the dark for 30 min at room temperature. Erythrocytes were then lysed for 10 min using the FACSLysis solution (Becton Dinckinson, Pont de Claix, France). The cells were subsequently washed twice with phosphate-buffered saline (Sigma, Saint Quentin Fallavier, France). For intracellular staining, a 1% paraformaldehyde-0.12% saponin technique was used. The monoclonal antibodies studied were CD45 phycoerythrin-Cy5 (PE-Cy5) (Beckman-Coulter, Marseille, France); fluorescein (FITC) directly conjugated CD2, CD5, CD10, CD15, CD36, CD38, CD41, CD42, CD61, CD71, and Glycophorine-A (Beckman-Coulter, Marseille, France); FITC directly conjugated CD3, CD22, CD34, TCRαβ and TCRγδ (Becton Dickinson, Pont de Claix, France); PE directly conjugated CD1a, CD7, CD13, CD19, CD24, CD33, CD34, CD56, and CD117 (Beckman-Coulter, Marseille, France); and PE directly conjugated CD11c (Dako, Denmark). They were combined as follows: CD36/CD11c/CD45, CD41/CD34/CD45, CD34/CD117/CD45, CD10/CD19/CD45, CD38/CD34/CD45, CD61/CD34/CD45, CD42/CD34/CD45, CD15/CD1a/CD45, CD71/CD24/CD45, Glycophorine-A/CD13/CD45, CD2/CD7/CD45, CD3/CD56/CD45, CD5/CD33/CD45. Intracellular staining was performed with CD3 (cCD3) and CD22 (cCD22) antibodies. Flow cytometric analysis was performed using an EPICS XL flow cytometer (Beckman-Coulter, Margency, France). Several populations were determined based on their CD45 expression on an SSC/CD45 histogram without previous back-gating, as previously described (9). Separate population phenotype analysis was performed.

RESULTS

Whole blood analysis on the SSC/CD45 histogram, revealed widely known populations: granular cells (G), monocytes (M), lymphocytes (L) and erythroblasts (E) (Fig. 1). In the blast cell location (CD45 dim, low side scatter), we observed two subpopulations labeled B1 and B2 (Fig. 2). Both were in equal proportion and represented about 2% of the entire blood cell population. They were CD34, CD117, and CD56 positive and no B-lymphoid surface and cytoplasmic antigens were found. However, the B2 population expressed immature antigens with myeloid commitment, i.e., CD38, CD71, CD33, and CD7. The B1 population had a lower CD45 expression, and was positive for megakaryocytic antigens, i.e., CD41, CD42, and CD61, as well as for CD13, CD36, CD1a, and CD2. Cytoplasmic and membrane CD3 (mCD3), TCRαβ TCRγδ and Glycophorine-A were negative in both subpopulations. No immunophenotypic abnormality was observed in monocytes or in lymphocytes that were of T-lineage (Table 1). On erythroblasts, we found coexpression of CD34, CD36, and megakaryocytic antigens (CD41, CD42, and CD61). Granular cells were positive for myeloid antigens (CD13, CD33, CD15, CD11c, CD24) as well as for CD1a and CD2, but cCD3, mCD3, CD7, and both TCR were negative.

DISCUSSION

Transient myeloproliferative disorder is found mainly in children with Down syndrome and is quite uncommon. Most of the immunophenotypic studies performed were made before the development of new flow cytometric immunophenotyping methods (1-3). A three color-flow cytometric method allowed us to distinguish two blast-cell subpopulations. The B2 subpopulation had a phenotype corresponding to the one described for immature myeloid progenitor with expression of CD34, CD38, and CD71, but also CD117, CD33, and CD7 (13).

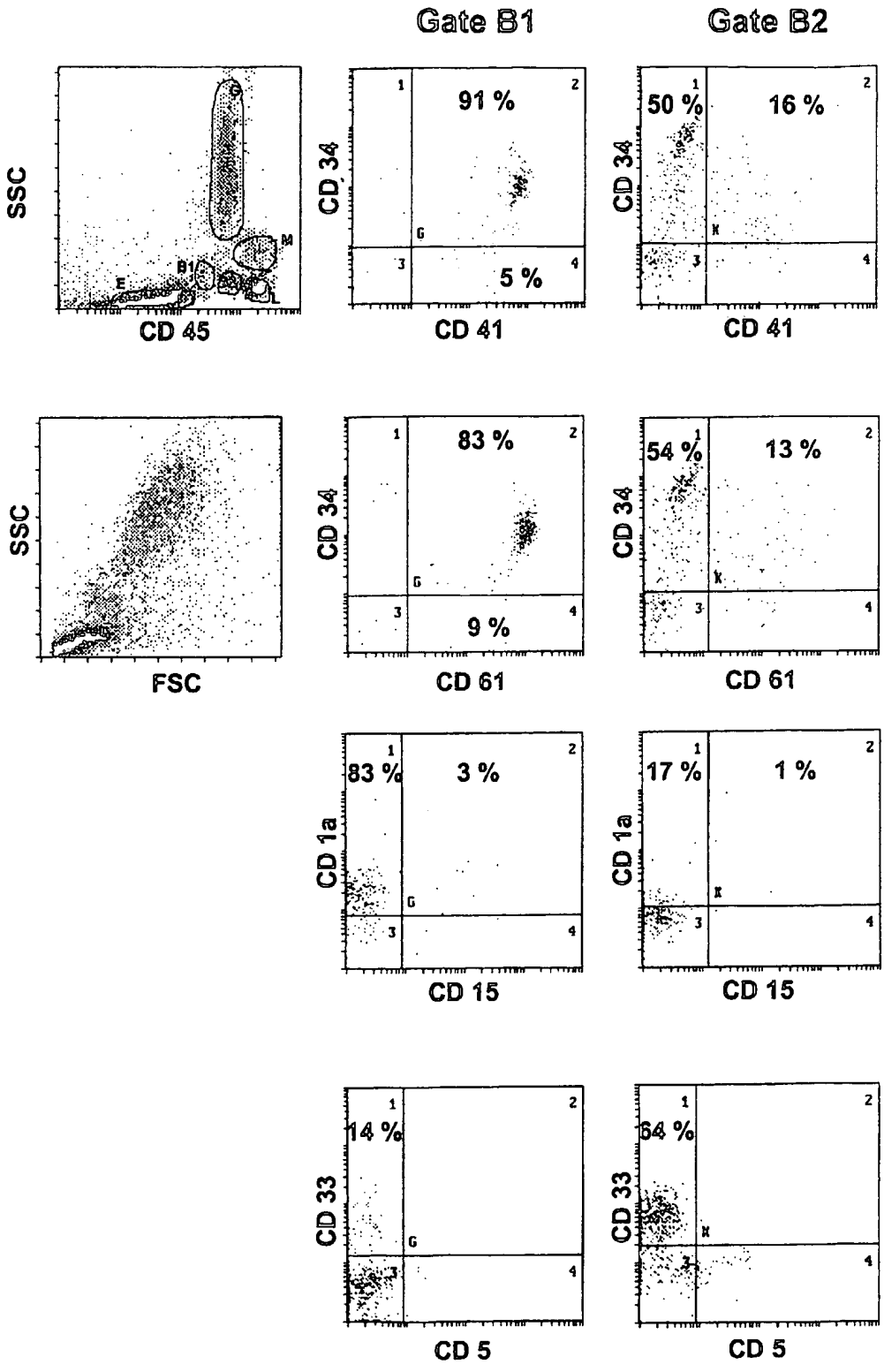


FIG. 2. Examples of double staining on the two CD45 dim blast cell subpopulations (B1 and B2). From top to bottom, the combinations shown are CD41(FITC)/CD34(PE); CD61(FITC)/CD34(PE); CD15(FITC)/CD1a(PE); CD5(FITC)/CD33(PE).

Table 1
Percentage of Positivity of Each Marker Tested on the 6 Populations Observed in the Analysis Based on SSC × CD45. mCD3 = CD3 on Membrane; cCD3 = CD3 Cytoplasmic.

	Blasts 1	Blasts 2	Granular cells	Erythroblasts	Lymphocytes	Monocytes
CD 1a	86	18	71	10	1	3
CD 2	83	18	90	1	91	8
TCR αβ	1	2	1	1	82	3
TCR γδ	0	0	0	0	1	0
mCD 3	4	13	3	1	88	2
cCD 3	6	4	2	1	90	2
CD 5	1	9	1	0	88	2
CD 7	15	80	3	0	97	8
CD 10	0	0	0	0	2	0
CD 11c	63	24	98	3	6	98
CD 13	88	16	99	13	0	97
CD 15	3	2	98	1	1	7
CD 19	0	0	0	0	2	0
CD 24	13	3	99	0	3	1
CD 33	14	64	99	0	1	97
CD 34	93	66	3	47	1	5
CD 36	87	44	5	98	3	97
CD 38	9	63	0	0	75	67
CD 41	96	18	4	98	1	7
CD 42	89	18	2	86	1	4
CD 56	90	71	24	90	10	7
CD 61	92	17	5	99	2	8
CD 71	17	64	2	2	1	36
CD 117	93	54	2	3	1	1
Glyco-A	2	12	1	1	0	2

The B1 subpopulation exhibited a phenotype with co-expression of three lineage antigens. Blast cells with multilineage antigens have been previously reported in such patients (6-8). We avoided false positive CD41, CD42, and CD61 expression by fluorescence microscopic examination. Megakaryocytic antigens have already been reported on TMD blast cells in a range of 75-80% of positive cells (1,3,11). Contamination by cells from gate E could be excluded because of the high percentage of positive cells observed and because of numerous phenotypic differences between these two populations. T-lineage commitment of TMD blast cells has also been reported based on cytoplasmic or membrane CD3 and CD7 expression (7,8,11,14). CD7 is quite common on immature myeloid cells—we found it on the B2 population but not on the B1 subpopulation. It could not then be considered of lymphoid-specific commitment. Absence of cCD3 expression and isolated expression of CD1a and CD2 was probably a phenotypic aberrance rather than a true T-lymphoid commitment. In previously reported cases, $CD3^+$ blast cells were very rare: mCD3 was negative in eight TMD, cCD3 was negative in one TMD, and cCD3 was positive in three of four cases of acute megakaryoblastic leukemia in Down syndrome (11,14). Molecular analysis was performed in only one $mCD3^+$ TMD and showed a clonal rearrangement of the T-cell receptor β (8). Normal immature CD34 positive/CD38 negative progenitor does not express lineage antigens that appear when CD38 becomes positive (5). On the B1 subpopulation, expression of CD117 and CD13 (which are of myeloid commitment) was observed. Furthermore, normal acquisition of CD33 and CD71 and loss of CD34, characteristic of the myeloid differentiation, was not found on this population (13). All these arguments lead us to consider that it was more likely an abnormal immature progenitor subpopulation. Expression of CD1a and CD2 on granular cells was also aberrant and suggests that they came from the B1 subpopulation.

On the cells of gate E we found expression of CD36 and of megakaryocytic antigens but Glycophorine-A was not yet expressed. This phenotype confirms the early erythroid progenitor nature of these cells, although CD71 was absent (10). We assume that expression of CD1a and CD13 on a few cells from gate E was due to contamination by the B1 subpopulation. Despite the spontaneous remission observed in this patient, a third of TMD patients develop an acute nonlymphocytic leukemia, mainly of M7-Fab subtype (AMKL; 4). Blast cells expressing three or four lineage antigens were frequently found in children with AMKL and Down syndrome (11). This suggests that an early progenitor could be involved in the leukemic process, and that the B1 subpopulation could be this progenitor. If, as it is postulated (15), TMD is the first step of a leukemic process, only large phenotypic analysis of the subsequent leukemic cells will enable this to be proven.

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2) Gabriel H.H.W. et al., Overtraining and immune system: A prospective longitudinal study in endurance athletes.
Medicine and Science in Sports and Exercise, (1998) 30/7 (1151-1157).

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CYTOMETRY, (1999 Nov 1) 37 (3) 178-83.

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SCANDINAVIAN JOURNAL OF HAEMATOLOGY, (1979 Jan) 22 (1) 25-32.

Thank you

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Cytometry 37:178–183 (1999)

Human Basophils Express CD22 Without Expression of CD19

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Background: Even modern automatic cell counters cannot count basophils precisely. Therefore, we need a rapid, accurate, precise, and easy method for counting basophils.

Methods: Using flow cytometry, basophils (CD22+/CD19-) and B cells (CD22+/CD19+) were counted. Within a large lymphocyte light scatter gate, % basophils (G%baso) and % B cells (G%B) were determined from the total count. Another method of analysis was to make two regions (R1 for basophils and R2 for B cells) and to determine in those the % basophils (R1%baso) and % B cells (R2%B) without gating. The flow cytometric basophil counts of the blood of 21 normal controls and 43 chronic myelogenous leukemia (CML) patients were compared with manual basophil count (Ma%baso) and basophil count by Coulter electronic cell counter (Hialeah, FL) (Auto%baso). CD22+/CD19- cells were sorted by a FACSCalibur (Becton Dickinson, San Jose, CA).

Results: The G%baso of all samples was 4.66 ± 5.35%, and R1%baso was 4.23 ± 4.88%, and they were well-correlated ($r = 0.996$, $P < 0.001$). The G%B of all samples was 1.55 ± 1.68%, and R2%B was 1.59 ± 1.67%, and they were also well-correlated ($r = 0.993$, $P < 0.001$). Their correlation was better in normal controls than in CML. G%baso was well-correlated to Ma%baso ($r = 0.827$) and Auto%baso ($r = 0.806$), and R1%baso was well-correlated to Ma%baso ($r = 0.831$) but showed poor correlation to Auto%baso ($r = 0.734$). Auto%baso revealed the poorest correlation to Ma%baso ($r = 0.692$). The sorted CD22+/CD19- cells were all basophils (99.48 ± 0.30%), and they revealed CD13, CD33, and dim CD45 expression, whereas CD3, CD14, CD16, and HLA-DR were not detected on them.

Conclusions: We discovered a specific marker combination to identify basophils (CD22+/CD19-), and we suggest that flow cytometric analysis using these markers is an easy, reliable, and accurate method of basophil counting. Cytometry 37:178–183, 1999. © 1999 Wiley-Liss, Inc.

Key terms: basophil; flow cytometry; CML; CD22; CD19

The human basophil is the least common granulocyte, with a prevalence of less than 1% of total leukocytes. The blood basophil counts are increased in all patients with Ph-positive chronic myelogenous leukemia (CML) (1–5), and basophilia is also found in other chronic myeloproliferative disorders (MPD), myelodysplastic syndrome, allergies, and inflammation (6–9). We demonstrated fusion of bcr and abl genes in the basophils of CML patients using fluorescent in situ hybridization (10). This means that the basophils belong to the CML clone. A rapid increase of the basophil count is one of the markers of impending acute transformation (1,2,6), and a negative correlation between basophil counts and survival in CML patients has been reported (11). Therefore, accurate and precise basophil counting is very important. Although the basophil has prominent metachromatic cytoplasmic granules, differential counts of blood films yield valid results only if the percentage of basophils is elevated or if many thousands of leukocytes are counted (12). Even modern automatic cell counters cannot count basophils precisely (13–15). There is no rapid, accurate, and precise method to count basophils in blood. The manual differential counting method is the method of choice, but it is laborious and it is very difficult to identify basophils accurately in CML patients, because of marked morphological variation (16,17). Furthermore, the basophils are distributed in the peripheral blood films irregularly (13). Correlations of automated and manual methods and even different automated methods are very poor (13,14). Recently, we identified a new set of markers which can identify basophils accurately and precisely using flow cytometry. This method can enumerate not only basophils but also normal B lymphocytes in the peripheral blood.

MATERIALS AND METHODS

Blood Samples

Peripheral blood from 21 normal individuals and 43 CML patients in chronic phase were collected into ethylene-

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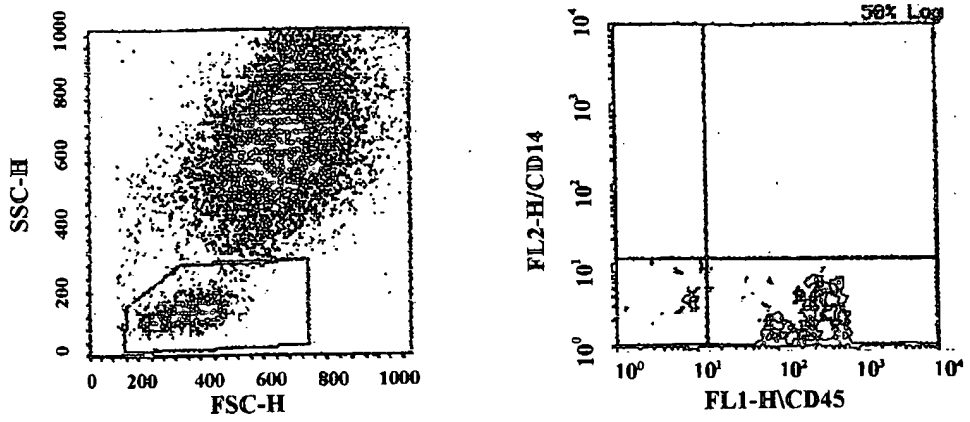

FIG. 1. Bivariate distribution of the peripheral blood cells of a CML patient displayed in a plot of forward scatter (linear scale) versus side scatter (linear scale), showing a large gate set around lymphocytes (left), and the gated cells displayed in a plot of FL1 (CD45-FITC) versus FL2 (CD14-PE) on a log scale showing typeical lymphocytes (high CD45 expression) and basophils (dim CD45 adjacent to lymphocytes (right).

diaminetetraacetic acid (EDTA)-vacutainer tubes. A case of breast cancer showing basophilia in the peripheral blood was also included. The samples were maintained at room temperature (18-20?) up to 1 h after collection before analysis. All samples were analyzed within 1 h after collection to avoid the possibility of degranulation of basophils.

Antibodies

All monoclonal antibodies were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Fluorescein isothiocyanate (FITC)-labeled anti-CD3, -CD19, -CD45, and -CD13 and Phycoerythrin (PE)-labeled anti-CD14, -CD16, -CD22, -CD33, and -HLA-DR were used.

Two-Color Immunofluorescence Study

Two-color Immunofluorescence studies were performed using whole blood. All samples were stained both with FITC- or PE-labeled monoclonal antibodies directed against CD3, CD13, CD14, CD16, CD19, CD22, CD33, CD45, and HLA-DR and with the negative isotype control antibodies. Then the erythrocytes were lysed by incubation in the lysing solution (Becton Dickinson) and the sediments were washed in phosphate-buffered saline (PBS). Fluorescence was analyzed by flow cytometry (FACScan, Becton Dickinson) using LYSYS version 1.1 software (Becton Dickinson). Instrument settings were controlled monthly using standard calibration beads (Becton Dickinson) and Autocomp software. Markers were set using isotype control sera, so that fewer than 1% of cells stained positively. Compensation was applied to correct for FITC emissions entering the FL2 channel and for PE emissions entering the FL1 channel. Results were recorded as the percentage of cells that stained positively and because the debris were CD45-, all data were corrected for debris contamination by multiplying 100/%CD45+. Basophils were identified by their positive staining for PE-conjugated CD22 monitored in FL2 (log scale), and their negative staining with FITC-conjugated CD19 monitored in FL1 (log scale). B cells were identified by CD22+/CD19+. We determined % basophil and % B cells using two different analysis methods. To get well-separated basophil and B cell signals, stained cells were displayed in a plot of forward scatter (linear scale) versus side scatter (linear scale), and a large gate was set around lymphocytes (Fig.1). Within this gate, % basophils (G%baso) and % B cells (G%B) were determined with respect to the total count (Fig.2), not from gated count, to get the basophil percentage of leukocytes in the blood. Another method of analysis was to count and calculate % basophils (R1%baso) and % B cells (R2%B) without any gating and as a substitute, make two regions for them (R1 for basophils and R2 for B cells) in the FL1 versus FL2 dot-plots, excluding adjacent clusters as much as possible (Fig. 3). To investigate the expression of other cell surface markers on the CD22+/CD19- cells, CML cases in which CD22+/CD19- cell populations were greater than 10 times the CD22+/CD19+ cell populations were selected, and two-color immunofluorescence studies using anti-CD22-PE- and FITC-conjugated anti-HLA-DR, anti-CD33, anti-CD2, anti-CD16, and anti-CD13 were performed.

Purification of CD22+/CD19− Cells Using Fluorescence-Activated Cell-Sorting Technique

Peripheral blood from 4 CML patients and 1 breast cancer patient with basophilia were collected into preservative-free heparin, and mononuclear cells were separated using Ficoll Hypaque (Sigma Chemical Co., St. Louis, MO) gradient centrifugation. The mononuclear cells were concentrated and stained with anti-CD22-PE and anti-CD19-FITC. Cells were analyzed on a FACS Calibur (Becton Dickinson) and then separated into CD22+/CD19- cells and other cells using the large lymphocyte gate. Cytospins were prepared from CD22+/CD19- cells and other cells on a cytocentrifuge (Shandon Southern Products Ltd., Cheshire, UK) and stained with Wright stain, which is one of the metachromatic stains. Differential counting of 500 cells per slide was performed using ×1,000 magnification, and photomicrographs were taken.

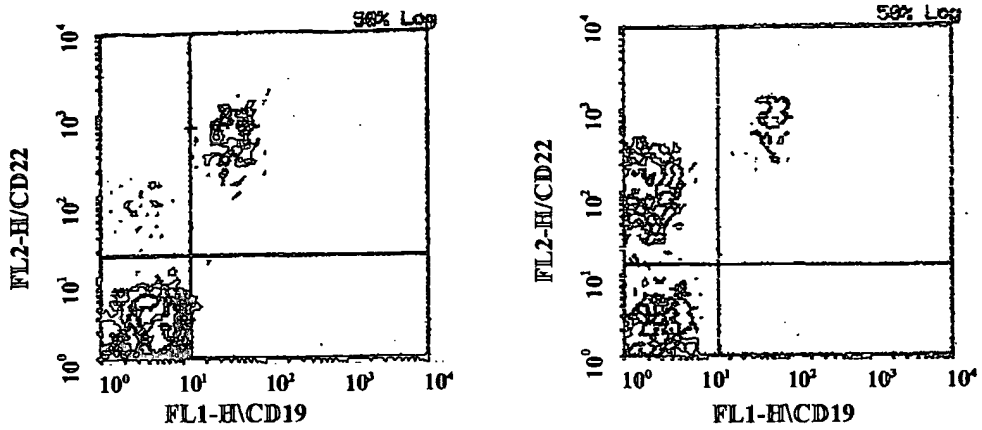

FIG. 2. Contour graphs of the cells in the large lymphocyte gate after staining with anti-CD 19-FITC (FL1) and anti-CD22-PE (FL2), showing basophils in the left upper quadrant (CD22+/CDE19−) and B cells in the right upper quadrant (CD22+/CD19+). **Left:** Contour graph from normal control, showing a larger population of B cells than basophils in contrast to the contour graph from a CML patient (**right**), showing a much larger population of basophils than of B cells.

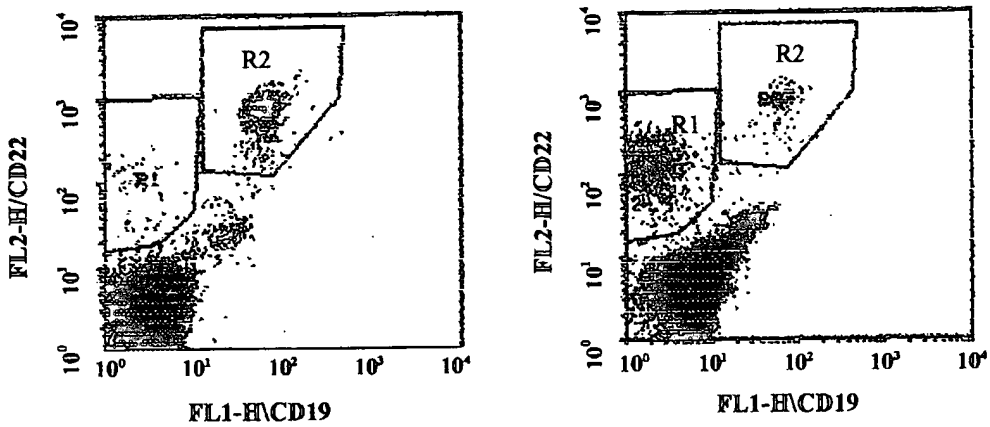

FIG. 3. Bivariate distribution of blood cells without gating after staining with anti-CD 19-FITC (FL1) and anti-CD22-PE (FL2), showing basophils in the R1 region (CD22+/CD19−) and B cells on the R2 region (CD22/CD19+). **Left:** Bivariate distribution from normal control, showing a larger population of B cells than basophils in contrast to that from a CML patient (**right**) showing a much larger population of basophils than of B cells.

Manual Basophil Counting

Manual leukocytes differentials were performed. The blood smears were prepared for each specimen within 1 h after collection and fixed and stained using the Wright stain. Four hundred cell differentials were performed by two technicians using suggestions given in the National Committee for Clinical Laboratory Standards (NCCLS) Tentative Standard H20-T (18), and % basophils (Ma%baso) was obtained. The cells showing severe morphological variations such as hypogranulation or mixed basophilic and neutrophilic/eosinophilic granules found in CML patients were reevaluated by a hematopathologist (K.H.).

Basophil Counting Using Coulter Electronic Cell Counter Model STKS

The Coulter (Hialeah, FL) STKS was installed and operated according to the manufacturer's instructions. EDTA blood samples were analyzed in the closed tube mode within 1 h of blood collection. The % basophil results are given as "Auto%baso."

Statistics

To evaluate correlations between cell counting methods, the Pearson correlation coefficient and *P* value were calculated. Significance was evaluated by the Wilcoxon signed rank test, using SPSS software (SPSS Inc., Chicago, IL).

RESULTS

The CD22+/CD19- cells showed dim CD45 expression as reported previously (19), and not all the cells in the gate expressed CD14 (Fig.1); the degree of CD22 expression was slightly dimmer than that of B lymphocytes (Figs. 2, 3). CD22+ cells revealed CD13 and CD33 expression on the dual immunophenotyping studies of CML cases, and the CD13+ and CD33+ cell populations were well-correlated

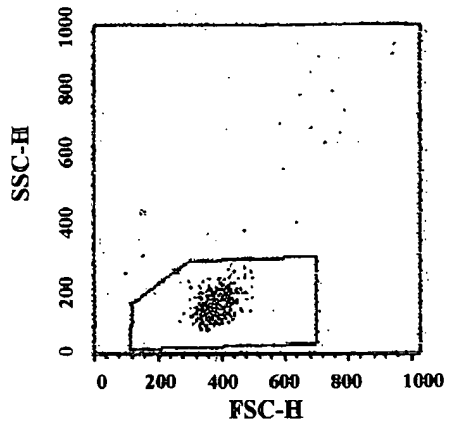

FIG. 4. The CD22+/CD19− cells of a CML patient were gated and displayed on the FSC and SSC scattergram. Most of the CD22+/CD19− cells were included in the large lymphocyte gate, and only a few CD22+/CD19− cells were distributed throughout the neutrophil and debris zone.

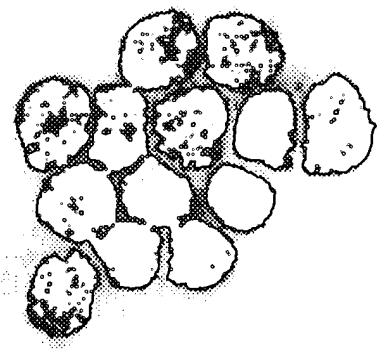

FIG. 5. Wright staining of the separated cells into CD22+/CD19− cell fraction on the FACS Calibur of a CML patient, showing pure basophils with typical large, dark granules.

to the CD22+/CD19- population, but HLA-DR, CD2, and CD16 were not expressed. The CD22+/CD19- cells were gated and displayed on the FSC and SSC scattergram. Most of the CD22+/CD19- cells (93.25 ± 2.27%) were included in the large lymphocyte gate, and only a few CD22+/CD19- cells were distributed throughout the neutrophil and debris zone in the CML patients (Fig. 4). The basophils of normal persons revealed the same distribution. The CD22+/CD19- cells sorted by the flow cytometer were basophils, and the purity was very good (99.48 ± 0.30%). They showed typical large, dark granules in their cytoplasms on Wright stain (Fig. 5). The morphology of the basophils was preserved well when they were mixed in fetal calf serum. The purity was not different between CML blood and the breast cancer patient's blood, although the basophils of CML patients showed fewer granules.

Results of basophil and B-cell counts by flow cytometry are given in Table 1. The gated basophil fraction of normal controls was well-separated from other cells, and G%baso was 0.89 ± 0.28%. R1 gating was also easy, and R1%baso was 0.89 ± 0.32%. G%baso and R_1%baso were almost the same and correlated well ($r = 0.984$, $P < 0.001$). The G%B of normal controls was 3.55 ± 1.43%, and R2%B was almost the same (3.55 ± 1.42%) and also correlated well ($r = 0.999$, $P < 0.001$). The B cells showed higher CD22 expression than did the basophils. The basophils of CML revealed a slightly variable degree of CD22 expression, contrary to normal persons showing the same degree of CD22 expression. However, it was also easy to gate basophils in CML patients. The G%baso of CML was 6.49 ± 5.69%, and was higher than R1%baso (5.86 ± 5.23%, $P < 0.001$). G%B was 0.57 ± 0.53% and was lower than R2%B (0.63 ± 0.59%, $P = 0.014$). However, they also correlated well ($r = 0.995$, $P < 0.001$ for % basophils, and $r = 0.925$, $P < 0.001$ for % B cells).

Percent of basophils by manual counting (Ma%baso) of all samples (64 cases) was 7.69 ± 9.79%, and that by Coulter STKS (Auto%baso) was 7.73 ± 9.70%. Manual basophil counts by technicians who were not experts in hematology were very variable. Hence, only basophil counting by experts was included in this study. Correlations between G%baso, R1%baso, Ma%baso, and Auto%baso are given in Table 2. G%baso was well-correlated to Ma%baso ($r = 0.827$) and Auto%baso ($r = 0.806$), and R1%baso showed better correlation to Ma%baso ($r = 0.831$) than to Auto%baso ($r = 0.734$). Auto%baso revealed poor correlation to other methods and the poorest correlation to Ma%baso ($r = 0.692$). However, there was no statistically significant difference between these basophil counting methods except between G%baso and R1%baso ($P < 0.001$, Table 2).

DISCUSSION

It is difficult to identify basophils in the peripheral blood smears of CML patients because of the simultaneous presence of eosinophilic and basophilic granules in the same granulocytes and because of granule atypicality (16,17). Since basophils constitute a small minority of peripheral blood cells, manual basophil counts are imprecise and the reference range is wide. Recently, all blood samples have been counted using automated cell counters. These cell counters have limitations in terms of counting basophils accurately, due mainly to false basophilia (15). A number of cells encountered as basophils by automated blood cell counters are blasts, lymphoma cells, and nucleated erythrocytes. Blasts and nucleated erythrocytes are frequently found in CML. Therefore, both manual basophil counts and automated blood cell counts have serious limitations. However, the basophil count is valuable in diagnosing and predicting the course of several diseases, including CML, allergies, and other conditions (2,11,20,21).

Basophils were recently investigated in order to identify specific markers on these cells. Basophils revealed dim CD45 expression and showed light-scattering patterns similar to those of lymphocytes on flow cytometry (2,22). Although basophils are one of the granulocyte subtypes, only some of the myeloid antigens are expressed, and they are known to have different phenotypes from other granulocytes such as neutrophils or eosinophils (6,23).

Table 1
Results of Basophil and B-Cell Counts by Flow Cytometry With and Without Gating, Using Antibodies to CD22 and CD19

	G%baso (mean ± SD)	R1%baso (mean ± SD)	Correlation coefficient (two-tailed)	G%B (mean ± SD)	R2%B (mean ± SD)	Correlation coefficient (two-tailed)
Normal control, N = 21	0.89 ± 0.28	0.89 ± 0.32	0.984 ($P < 0.001$)	3.55 ± 1.43	3.55 ± 1.42	0.999 ($P < 0.001$)
CML, N = 43	6.49 ± 5.69	5.86 ± 5.23	0.995 ($P < 0.001$)	0.57 ± 0.53	0.63 ± 0.59	0.925 ($P < 0.001$)
Total, N = 64	4.66 ± 5.35	4.23 ± 4.88	0.996 ($P < 0.001$)	1.55 ± 1.68	1.59 ± 1.67	0.993 ($P < 0.001$)

*G%baso, percent of basophils by the large lymphocyte gating method; R1%baso, percent of basophils by the method without gating (% region 1 cells); G%B, percent of B cells by the large lymphocyte gating method; R2%B, percent of B cells by the method without gating (% region 2 cells).

Table 2
*Results of Statistical Analysis of Basophil Counting Methods**

	G%baso vs. R1%baso	G%baso vs. Ma%baso	R1%baso vs. Ma%baso	G%baso vs. Auto%baso	R1%baso vs. Auto%baso	Ma%baso vs. Auto%baso
Correlation r value	0.996	0.827	0.831	0.806	0.734	0.692
Wilkoxon signed rank test P value (two-tailed)	<0.001	0.886	0.274	0.911	0.775	0.896

*G%baso, percent of basophils by flow cytometry with the large lymphocyte gating method; R1%baso, percent of basophils by flow cytometry without gating (% region 1 cells); Ma%baso, percent of basophils counted manually; Auto%baso, percent of basophils by Coulter STKS.

T-cell activation markers such as CD25, leukocyte adhesion molecules such as CD11b/18, receptors for IgG, BSP-1, IgE, IL-3Rα (CD123), and several other molecules have been identified on basophils (6,17,22-29). But the kinds of antigens expressed on basophils are still a matter of controversy (23,24,27,30). This could be due to paucity of basophils in the peripheral blood. Also, flow cytometry has not been successful in replacing manual basophil counting until now (11). We observed that the proportion of CD22+ cells exceeded the CD19+ cell population in the peripheral blood of CML patients, in contrast to normal controls who showed the same proportion, and we found that the size of difference correlates to the basophil count. Further, the CD22+ cells in CML patients who had negligible CD22+/CD19+ cells showed CD13 and CD33 expression. Because most of the CD22+ cells were basophils, basophils express CD13 and CD33. These results are the same as in previous reports (24,31). Most of the CD22+/CD19- cells were included in the lymphocyte gate at a slightly greater FSC than small lymphocytes. These CD22+/CD19- cells were sorted, and they were all basophils (purity, 99.48%). Several attempts to purify human basophils have been reported (23,29,31). However, the method described here is easier and revealed higher purity. Therefore, we recommend CD22+/CD19- cell sorting using a flow cytometer for basophil purification and research about human basophils.

CD22 (BL-CAM) has been shown to be expressed on precursor and mature B cells only, and not on any other blood cells (32). Agis et al. (24) performed comparative phenotypic analyses on basophils using monoclonal antibodies to many CD antigens (CD1-130) and reported that basophils did not express CD22 on their surfaces. This could have been due to paucity of basophils in the sample (3.5-6.7%) or to misinterpretation of all CD22-positive cells as B lymphocytes. In other words, because basophils are included in the lymphocyte gate and nobody has thought CD22 to be expressed on blood cells other than B cells, CD22 expression on basophils has been obscured. Recently, Toba et al. (31) also reported that basophils do not express CD22. It is possible that because they used unconjugated monoclonal antibody to CD22 and fluorochrome-conjugated secondary antibody, the basophils which showed dimmer CD22 expression than normal B lymphocytes could have been misinterpreted as CD22 negative.

We compared two methods of flow cytometric analysis. One method was to get data from a large gate around lymphocytes. The advantage of this method is well-separated signals. But its weak point is the need for a troublesome gating step and the possible escape of basophils from this gate due to their morphologic variations. Another method of analysis was to read data from regions directly. Although it is not easy to make regions for common use, this method would be better for automation. The basophil and B-cell counts of normal controls obtained by these two methods were almost the same. But R1%baso was smaller than G%baso in the CML group ($P < 0.001$). Because the basophils in CML show a wide range of morphologic variation, they could have a wide range of antigen density on their surfaces, and the distribution of basophils could be wider than in normal controls. Although flow cytometric basophil counts and manual basophil counts were not significantly different, Ma%baso was closer to G%baso than to R1%baso. Therefore, we regarded the difference between R1%baso and G%baso as an artifact due to the small size of region R1. Maybe we

should make a slightly bigger region for basophilia samples such as CML. However, the counts using these two methods were very well-correlated (r = 0.996). Correlations of these flow cytometric basophil counts to manual counts and automated basophil counts were better than between manual and automated basophil counts. And there was not a significant difference among these basophil counts ($P > 0.1$) except between two flow cytometric methods. Therefore, we conclude that all the basophils in the CML patients or normal controls expressed CD22 and not CD19 and therefore were CD22+/CD19-.

CD19 is a well-known marker of B cells in all stages of maturation. Therefore, the combination of CD22 and CD19 made it possible to count not only basophils but also B cells. B cells showed high CD45 expression and expression of both CD22 and CD19. The number of B cells in the peripheral blood is useful in many diseases including hypogammaglobulinemia, and after bone marrow transplantation. And from this study, we found that the basophils could be counted as dendritic cells because both cells are included in the mononuclear cell region and do not express lymphocyte or monocyte antigens such as CD2, CD14, CD16, and C19. Generally, CD22 is not included in the lymphocyte panel. Therefore, the most useful antigen which can discriminate basophils from dendritic cells in the peripheral blood is HLA-DR, because the dendritic cells express high HLA-DR antigen in contrast to basophils, which do not express this antigen on their surface (33,34).

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Flow cytometric analysis of the effect of dithiothreitol on leukocyte surface markers

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ABSTRACT: Pretreatment with dithiothreitol (DTT) is necessary to dissolve mucus in samples of induced sputum prior to analysis. However, DTT may affect cell surface markers which are essential for lymphocyte subtyping. Therefore, the aim of this study was to evaluate the effect of DTT on an appropriate panel of surface markers. Peripheral blood leukocytes were used because these cells, in contrast to sputum cells, could be obtained without DTT treatment.

Peripheral blood from healthy donors was incubated with either DTT according to standard sputum procedures or phosphate-buffered saline (PBS), washed and incubated with fluorochrome-labelled antibodies. After lysis of erythrocytes, analysis was performed using a calibrated flow cytometer. Leukocyte populations were identified by their light scattering properties. For analysis, fluorescence intensity was compared between DTT- and PBS-treated samples.

After treatment with DTT, fluorescence intensity was significantly increased in CD16-positive granulocytes; it was reduced in CD2-positive lymphocytes, CD45-positive lymphocytes and CD14-positive monocytes ($p \leq 0.001$). These changes occurred in all samples. The fluorescence intensity of CD3-, CD4-, CD8-, CD19-, CD56- and histocompatibility leukocyte antigen DR-positive lymphocytes was not altered by DTT. However, there were statistically significant ($p<0.001$), although small, changes in the percentages of leukocytes.

The present data demonstrate that, although dithiothreitol as used in sputum analysis affects some surface markers of peripheral blood leukocytes, comparability between samples concerning lymphocyte surface markers is preserved. Therefore, it is suggested that treatment of sputum samples with dithiothreitol does not invalidate the immunocytochemical analysis of lymphocytes.

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The method of induced sputum is widely used as a non-invasive procedure for obtaining biological samples from the airways [1, 2] in order to determine their cellular and biochemical composition in relation to airway diseases [3, 4]. As sputum cells are embedded in airway secretions, the material has to be liquified in order to prepare single-cell suspensions for flow cytometry. This can be achieved by incubation with the potent reducing agent dithiothreitol (DTT, 2,3-dihydroxybutane-1,4-dithiol) [5]. Therefore, DTT is widely used in sputum processing [6–9]. However, owing to its reducing properties, DTT could affect the three-dimensional structure of proteins, which is maintained by disulphide bonds, thereby leading to changes in the availability and integrity of epitopes which could hamper immunological detection procedures [10].

Indeed, the data which are available indicate effects of DTT or dithioerythritol (DTE) on surface markers assessed by flow cytometry on eosinophils or neutrophils even if cell viability remains unchanged [11–13]. Data regarding markers that are essential for lymphocyte subtyping have been published in abstract form [14]. Recent data have shown improved cell and sputum supernatant inflammatory mediator recovery when using DTE [15]. As lymphocytes are important players in airway diseases, the study of these cells in induced sputum deserves particular attention [10, 16, 17]. Therefore, in the present study a detailed analysis of the effects of DTT on the appropriate surface markers was performed, and careful calibration using fluorescent beads included to improve the identification of those changes in cellular distribution that were due to mere reductions in fluorescence intensity. This appears to be particularly important in the analysis of lymphocyte subpopulations, because it supports the validation of data by the computation of check sums [17, 18].

Therefore, the aim of the present study was to evaluate the effect of DTT on the detection of a specific panel of leukocyte surface markers. As it was important to study the effect of DTT in cells which had not been previously treated with this compound, it was decided to investigate peripheral blood leukocytes as a model for induced sputum cells.

Material and methods

Subjects

Peripheral blood was obtained from 15 volunteers (10 male, five female; age 20–45 yrs) who showed a normal

white blood cell count and were judged healthy on a clinical basis. Blood was taken by venous puncture in the morning.

Processing

Samples of ethylenediamine tetra-acetic acid anticoagulated whole blood (100 µL) were incubated with either 250 µL Sputolysin® (0.1% or 6.5 mM dithiothreitol in 100 mM phosphate buffer, pH 7.0; Calbiochem, Bad Soden, Germany) according to a standard sputum procedure (final concentration 0.07% DTT) [19, 20] or 250 µL phosphate-buffered saline (PBS) for 30 min at 37°C. Afterwards, samples were washed twice with 4 mL PBS and centrifuged for 5 min at 300×*g*. After removing the supernatant, cells were incubated for 10 min at room temperature (25°C) with different amounts of fluorescence-labelled antibodies (table 1) according to the manufacturers' instructions. After lysis of erythrocytes with Immunoprep® on a Coulter® Multi-Q-Prep (Coulter Electronics GmbH, Krefeld, Germany), flow cytometric measurements were performed under standardized conditions using a four-colour Coulter® EPICS® XL-MCL equipped with one 488-nm argon laser. All measurements were performed in duplicate to assess intraassay variability. On average (minimum), 8,698 (4,122) granulocytes were counted for CD16, 1,526 (497) monocytes for CD14 and 4,850 (1,337) lymphocytes for all other markers.

Analysis

Leukocyte populations were identified by their light-scattering properties using logarithmic sideward scatter *versus* linear forward scatter (fig. 1). This approach was chosen instead of the combination of CD45 expression and sideward scatter in order to maintain the same gating strategy for all surface markers, independently of potential alterations in CD45 expression. The population of monocytes, as identified morphologically (fig. 1, gate C), was selected for the determination of CD14, that of granulocytes (fig. 1, gate A) for CD16 and that of lymphocytes (fig. 1, gate B) for all other surface antigens studied (table 1). The numbers of blood samples studied for each antibody are given in table 2, the variation in numbers arising from different availability of donors; it was not due to technical problems.

Table 1. – Antibodies and target cells used

Antibody conjugate	Clone	Target as used in this study	Manufacturer
CD14-PE	RMO22	Monocytes	Immunotech*
CD16-PE	3G8	Granulocytes	Immunotech*
CD19-FITC	SJ 25-C1	B-lymphocytes	Caltag+
CD2-FITC	39C1.5	T-lymphocytes	Immunotech*
CD3-FITC	UCHT1	T-lymphocytes	Immunotech*
CD4-PE	13B8.2	Helper/inducer T-lymphocytes	Immunotech*
CD45-FITC	J.33	Leukocytes (lymphocytes)	Immunotech*
CD56-PE	B159	NK cells	Immunotech*
CD8-PE	B9.11	Cytotoxic/suppressor T-lymphocytes	Immunotech*
HLA-DR-FITC	I3O	Activated lymphocytes	Coulter*

*: *via* Coulter-Immunotech, Hamburg, Germany; +: *via* medac, Hamburg, Germany. FITC: fluorescein isothiocyanate (fluorescence detector (FL) 1); PE: phycoerythrin (FL2); HLA: histocompatibility leukocyte antigen; NK: natural killer.

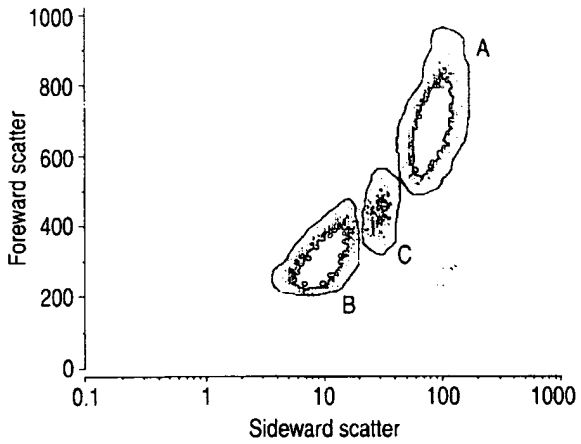

Fig. 1. – Identification of peripheral blood leukocyte population by their light scattering properties. Foreward scatter (linear) is related to cell size, sideward scatter (logarithmic) to granularity. Small mononuclear lymphocytes are shown in gate B, monocytes in gate C and polymorphonuclear granulocytes in gate A.

The flow cytometer was calibrated using DAKO-FluoroSpheres (DAKO Diagnostika GmbH, Hamburg, Germany), which consisted of blank beads and calibration beads. The latter comprised five bead populations of the same particle size labelled with different amounts of fluorochrome. After excitation by a 488 nm argon laser, the beads emitted signals of different fluorescense intensity which appeared in all fluorescence detectors (FL) 1–4 of the flow cytometer. The calibration-beads had been calibrated against soluble fluorochromes such as fluorescein isothiocyanate (FL1), phycoerythrin (PE; FL2), or a PE/cyan-5 tandem conjugate (FL4) by the manufacturer. For each fluorescence colour, a calibration function was constructed in order to express signal intensity (mean channel) as the number of molecules of equivalent soluble fluorochrome (MESF). The signal of the blank beads was used to determine the level of electronic noise and the limit of detection. This standardized procedure accounted for variations in the performance of the equipment and ensured that results showed optimal reproducibility.

Statistical analysis

Throughout the analysis, means of duplicate samples were used. For the comparison of cell differentials, the individual means of the 15 subjects were taken. Cell percentages and MESF were expressed as mean±SEM. Changes in fluorescence intensity caused by DTT were expressed as mean±SD percentage differences between the DTT and PBS values relative to the PBS values. The paired t-test was used to compare DTT and PBS treatment.

Table 2. – Intraclass correlation coefficients percentages of antibody-positive cells and fluorescence intensity in dithiothreitol (DTT)- and phosphate-buffered saline (PBS)-treated duplicate samples

Cell types	Ancestor	Samples n	Antibody-positive cells %				Fluorescence intensity MESF			
			Ri		PBS	DTT	Ri		PBS	DTT
			PBS	DTT			PBS	DTT		
Monocyte	Leukocyte	13	0.92	0.89	7.55±0.57	9.68±0.71**	–	–	–	–
Granulocyte	Leukocyte	15	0.94	0.99	61.71±1.87	64.41±1.90**	–	–	–	–
Lymphocyte	Leukocyte	15	0.99	0.98	32.75±1.21	29.32±1.55**	–	–	–	–
CD14-pos	Monocyte	13	0.92	0.17+	76.14±2.61	79.10±1.54	0.97	0.82	185896±6547	172263±5607**
CD16-pos	Granulocyte	15	0.96	0.99	93.26±1.01	94.46±0.99**	0.92	0.91	365269±22470	418443±24517**
CD19-pos	Lymphocyte	10	0.99	0.90	10.32±1.20	9.94±1.08	0.88	0.87	22176±568	21874±564
CD2-pos	Lymphocyte	13	0.47+	0.98	76.72±1.85	73.00±2.04*	0.99	0.87	28938±775	16387±348**
CD3-pos	Lymphocyte	10	0.99	1.00	70.82±2.50	66.49±3.07*	0.95	0.97	97063±3295	95965±3160
CD4-pos	Lymphocyte	10	0.99	0.73	41.51±1.90	36.97±2.42*	0.82	0.88	48154±2755	46898±2853
CD45-pos	Lymphocyte	12	0.89	0.93	96.46±0.35	94.96±0.55*	0.98	0.97	149545±2319	147558±2317**
CD56-pos	Lymphocyte	14	0.96	0.98	13.52±1.48	14.61±1.72*	0.72	0.83	8441±284	8583±350
CD8-pos	Lymphocyte	10	0.98	0.99	24.49±1.90	23.45±1.99*	0.96	0.97	87452±3229	89764±3176
HLA-DR-pos	Lymphocyte	12	0.69	0.88	13.40±0.60	15.06±0.63*	0.95	0.94	148021±8717	148432±8570

Data are presented as absolute values or as mean±SEM. +: due to outliers (see *Results* section); MESF: molecules of equivalent soluble fluorochromes; Ancestor: reference cell population; Ri: intraclass correlation coefficient; pos: positive; HLA: histocompatibility leukocyte antigen. *: $p<0.05$; **: $p<0.01$.

Statistical significance was assumed at a first kind error of $p<0.05$. Intra-assay reproducibility was derived from the duplicate samples by one-way analysis of variance using the intraclass correlation coefficient (Ri). In a similar manner, the effect of DTT *versus* PBS was expressed in terms of Ri.

Results

As compared to PBS, treatment with DTT caused statistically significant changes in standard cell differentials. After DTT treatment, the flow cytometer showed, on average, 2.1% more monocytes, 2.7% more granulocytes and 3.4% less lymphocytes (table 2; all $p<0.001$). Corresponding Ri indicated high reproducibility of differential cell counts (table 2). However, there were two outliers in the case of CD14-positive monocytes (after DTT) and one outlier in the case of CD2-positive lymphocytes (after PBS).

The percentages of antibody-positive cells showed statistically significant differences (all $p<0.05$) between samples treated with PBS or DTT (table 2). After DTT treatment, the percentages of CD16-positive granulocytes, and of CD56- and histocompatibility leukocyte antigen (HLA)-DR- positive lymphocytes were increased by 1.2, 1.1 and 1.7%, respectively; those of CD2-, CD3-, CD4-, CD45- and CD8-positive lymphocytes were reduced by 3.7, 4.3, 4.5, 1.5 and 1.1%, respectively. The differences in CD14-positive monocytes (3.0%) and CD-19-positive lymphocytes (-0.4%) were not statistically significant. The reproducibility of duplicate samples (Ri) ranged 0.69–1.0, except for CD14-positive monocytes after DTT and CD2-positive lymphocytes after PBS, where Ri were <0.5 (table 2). Effects of DTT in relation to PBS were also visible in terms of low Ri (table 3), particularly for CD14, CD2, CD45 and HLA-DR.

Regarding fluorescence intensity, treatment with DTT led to a statistically significant (all $p \leq 0.001$) increase in CD16-positive granulocytes, and to a reduction in CD2-positive lymphocytes, CD45-positive lymphocytes and CD14-positive monocytes (table 2). No statistically significant changes occurred in CD3, CD4, CD8, CD19, CD56 and HLA-DR. Differences between duplicate samples showed Ri of >0.7 (table 2). Figure 2 illustrates the mean effect of DTT on fluorescence intensity. A comparison of DTT- and PBS-treated samples in terms of Ri is given in table 3, demonstrating a marked effect on CD2.

Discussion

The present data demonstrate that, for a number of surface markers, DTT alters the fluorescence intensity of immunostained leukocytes as detectable by flow cytometry.

Table 3. – Intraclass correlation coefficients for percentages and fluorescence intensities between dithiothreitol- and phosphate-buffered saline-treated samples.

Cell type	Ancestor	Ri	
		Percentage	FI
CD14-positive	Monocyte	0.58	0.77
CD16-positive	Granulocyte	0.91	0.82
CD19-positive	Lymphocyte	0.95	0.90
CD2-positive	Lymphocyte	0.54	-0.84
CD3-positive	Lymphocyte	0.77	0.97
CD4-positive	Lymphocyte	0.70	0.83
CD45-positive	Lymphocyte	0.40	0.96
CD56-positive	Lymphocyte	0.96	0.81
CD8-positive	Lymphocyte	0.97	0.91
HLA-DR-positive	Lymphocyte	0.39	0.97

Ri: intraclass correlation coefficient; Ancestor: reference cell population; FI: fluorescence intensity; HLA: histocompatibility leukocyte antigen.

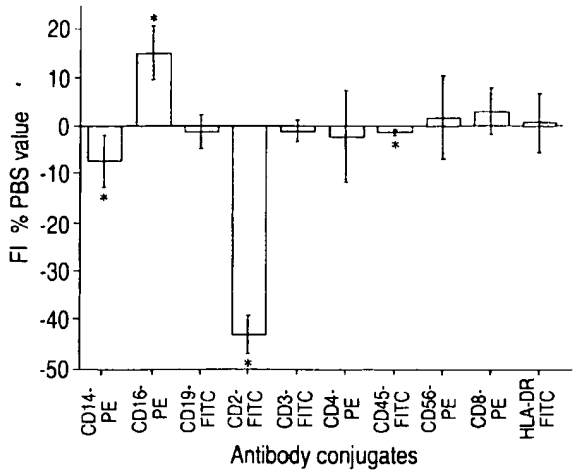

Fig. 2. – Effects of dithiothreitol (DTT) on flurescence intensity (FI). Changes in FI caused by DTT were expressed as mean±SD percentage differences between the DTT and phosphate-buffered saline (PBS) values relative to the PBS values. Samples were analysed in duplicate and numbers of subjects were as in *table 2*. PE: phycoerythrin; FITC: fluorescein isothiocyanate; HLA: histocompatibilty leukocyte antigen. *: $p<0.05$ *versus* PBS.

However, most changes were small and well within the range of variability of measurements. Furthermore, all studied cell populations remained well above the detection limit.

The present study was undertaken to elucidate the potential effect of DTT in sputum analysis. Sputum cells are embedded in a matrix of airway secretions produced by mucosal epithelial cells and submucosal glands [21]. The major component of this are mucins or mucus glycoproteins, which comprise up to 1–3% of sputum wet weight [22, 23]. These components are primarily responsible for the viscosity, elasticity and adhesive capacity of mucus [24]. In order to analyse sputum cells, it is necessary to extract them from their matrix. This can be achieved by destruction of the macromolecular structure of mucins. As this is maintained by disulphide bonds, cleavage of these bonds is one approach to dissolving sputum samples. Pretreatment of sputum samples by appropriate reducing agents such as DTT is most effective and corresponding cytospin preparations yield results which are more reproducible than, for example, sputum smears [25].

The effectiveness of DTT arises from the fact that the cyclic disulphides formed within DTT are energetically favoured and more stable than noncyclic disulphide bonds [26]. However, by the same mechanism as for mucus glycoproteins, DTT could also affect the three-dimensional structure of membrane proteins. These effects could hamper immunological detection procedures based on specific antibodies and cause a decrease in antibody binding. As a consequence, fluorescence intensity, as measured by flow cytometry, could be severely altered.

Owing to the fact that the aim was to reveal the effect of DTT, cells that could be obtained without DTT pretreatment, with minimum likelihood for potential cell loss, had to be used. Other methods of mucus liquification, such as the use of a needle [11], repeated washing [12] or incubation with an enzyme mixture [13] might affect the integrity of cells and lead to selective recovery. Therefore, it was decided to use peripheral blood leukocytes that could be obtained with as few interventions as possible as a model for sputum cells. It has been suggested that the expression of surface markers, *e.g.* CD11b and CD18, is about three times higher in the peripheral blood of healthy donors as compared to sputum cells from patients with bronchiectasis [12]. The fact that, in the present data, the mean fluorescence was always >8,000 MESF and the limit of detection was <1,000 MESF renders it unlikely that, even with signals of half the intensity (as would be expected for induced sputum), the detection limit would be reached.

At least theoretically, the presence of mucus could affect immunological detection, *e.g.* by absorption of antibodies. However, the present data would remain valid if the concentration of DTT to which sputum cells are exposed were markedly reduced by interaction with mucins. Certainly some proviso is needed before extrapolating the present data to induced sputum. However, the arguments outlined above suggest that immunological staining for flow cytometry can be performed successfully in sputum cells which, before staining, have been necessarily separated from mucus.

The flow cytometric analysis of lymphocyte subsets in induced sputum is of particular interest. It requires antibodies directed against CD2, CD3, CD4, CD8, CD14, CD16, CD19, CD45, CD56 and HLA-DR. Until now, data referring to the effect of DTT on these markers have only been available in abstract form. The study reported that DTT did not alter the detection of CD3, CD4, CD8, CD14, CD19, CD25 and CD45 in blood leukocytes but led to a significant reduction in HLA-DR by 18% [14]. It remained unclear as to whether percentages of positive cells or fluorescence intensities had been evaluated. A later study which utilized flow cytometry and incubation with DTT showed that sputum lymphocytes differed between smokers and asthmatic subjects; however, this study did not investigate the effect of DTT [10].

Other data on the effect of DTT on immunological cell detection refer to surface markers which are not primarily important for the analysis of lymphocytes. As compared to samples prepared by repeated washing, treatment with 0.1% DTT caused a reduction in the mean fluorescence intensity of CD11b and CD18 in the sputum of patients with bronchiectasis and in the peripheral blood neutrophils of healthy donors [12]. In accordance with this, DTE caused a significant reduction in detectable levels of blood cosinophil CD11a, CD11b and CD18, whereas CD9, CD11c, CDw32 and CD35 were not affected [11].

As a result of these considerations, the effect of DTT on the flow cytometric detection of that panel of surface markers which is essential for lymphocyte subtyping was evaluated. To achieve optimal precision, its effect on the percentages of cells as well as on fluorescence intensity under strictly standardized conditions of calibration and quality control was assessed.

With the exception of CD14-positive monocytes and CD19-positive lymphocytes, the percentages of antibody-positive cells differed significantly between PBS- and DTT-treated samples. This was reflected in low Ri particularly for CD2, CD45 and HLA-DR (table 3). CD14 also showed a low Ri, despite the fact that the difference between DTT- and PBS-treated samples was not significant.

It should be noted, however, that, by its definition, the Ri does not contain all information regarding the comparison of DTT- and PBS-treated samples. As blood from subjects with normal white blood cell counts was investigated, the between-subjects variation was rather low as compared to the within-subjects variation, and that might have biased the Ri. Regarding the percentages of antibody-positive cells, duplicate samples, which were incubated separately, showed high Ri, thereby indicating high intra-assay reproducibility for most surface markers. The outliers may have occurred because the amount of antibody used for incubation was too small or because the vortex agitation of the sample was insufficient.

Regarding fluorescence intensity, all duplicate samples showed acceptable reproducibility. In CD2- and CD45-positive lymphocytes and CD14-positive monocytes, the fluorescence intensity was significantly reduced in DTT- as compared to PBS-treated samples. The authors suggest that epitopes are altered by DTT in such a way that the number of antibodies bound to the cell surface is reduced. Conversely, an increase in fluorescence intensity, as observed for CD16, might be due to the fact that changes in the adjoining structure expose epitopes. For HLA-DR, the present data differ from those of KIDNEY *et al.* [14] who found a reduction of 18%. However, it is difficult to compare the results between studies as these authors did not give information about their method of data quantification.

Within the present data, the reduced fluorescence intensity of CD45- and CD2-positive lymphocytes was consistent with a lower percentage of lymphocytes, and the increased fluorescence intensity of CD16-positive granulocytes with a higher percentage of granulocytes. In contrast, it is difficult to reconcile the reduction in fluorescence intensity of CD14-positive monocytes after treatment with DTT, with the concomitant increase in the percentage of monocytes. In both cases, the effect of DTT on fluorescence intensity was small as reflected in high Ri, with the exception of CD2 (table 3). It is noteworthy that the differences in fluorescence intensity between samples treated with DTT and PBS were not likely to be the cause of changes in the percentages of antibody-positive cells because positive and negative cells were always easily distinguishable.

The percentages of morphologically identifiable leukocytes differed slightly but significantly between PBS- and DTT-treated samples. The mechanism by which DTT caused these effects remains unclear; possibly they were linked to the lysis of erythrocytes which is not necessary for analysis of induced sputum. Ri for duplicate samples indicated higher reproducibility after DTT than after PBS. It should be noted, however, that the shifts in leukocyte numbers were well within the range of flow-cytometric accuracy.

The results of the present study were obtained in healthy subjects, and extrapolation to other groups, for example subjects with asthma, remains to be established. In addition, further investigations on the relationships between the state of activation and surface markers in leukocytes from blood and sputum, and on the influence of mucins would be helpful. Despite these limitations, however, the present study demonstrates that, with regard to percentages of antibody-positive cells, the effects of DTT are likely to be small.

In conclusion, dithiothreitol at the concentrations used in sputum processing affected some surface markers of peripheral blood leukocytes, with respect to both fluorescence intensity (CD2, CD14, CD16, CD45) and percentages of antibody-positive cells. However, the analysis suggests that dithiothreitol does not invalidate the comparison between different samples regarding the immunocytochemical analysis of lymphocyte surface markers.

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Sequential Flow Cytometric Analysis of Cellular DNA-Content in Peripheral Blood during Treatment for Acute Leukaemia

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Sequential flow cytometric analysis (FCM) of relative nuclear DNA content per cell was done in peripheral blood of 12 patients during treatment for acute leukaemia. A marked increase of cells with S-phase DNA-content during the first hours of treatment was found in patients responding favorably to treatment. One patient with increase of 'S-phase cells' died before clinical improvement could be evaluated. However, lack of S-phase increase at one treatment cycle did not exclude a favorable response in the next. Two cases with probable aneuploid leukaemia showed gradual disappearance of abnormal cells during therapy. The value of FCM analysis of peripheral blood seems to be in predicting the response to treatment before clinical signs appear.

Key words: acute leukaemia – flow cytometry – peripheral blood

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Flow cytometry (FCM) is a rapid method for determining the proliferative pattern of a cell population by measuring the relative DNA content in the different cell cycle phases. Recently the method has been used in haematology during treatment for acute leukaemia. Most of these studies have been done on bone marrow (Büchner et al 1974, Hillen et al 1975a/b, Smets 1976), few on peripheral blood (Melamed 1973). A single cytostatic agent regimen, which has been used in most of these investigations, has the advantage that the site of action of the drug can be determined, especially when combined with other methods for cell cycle analysis. However, it is generally accepted that multiple drug regimens given at intermittent cycles increase the remission rates.

A practical and ethical problem when trying to follow the early effects of treatment with bone marrow samples is the difficulty in obtaining more than one or possibly two samples a day, and this is not sufficient for a reliable sequence of cell

TABLE 1

Therapy schemes used in acute leukaemia
Main effects of the drugs on the progression through cell cycle as well as main phase of cytotoxic effect
Compiled from Clarysse et al (1976) – Doses in mg

	Day / Dose				Cell cycle phase inhibition	Cytotox. phase
	1	2	3	4		
PRAP						
6-mercaptopurine	150	150	150	150	S	
Rubidomycin	60				S	S
Ara-C	150	150	150	150	S	S
Prednisone	10×4	10×4	10×4	10×4	G_1	G_1
COAP						
Cyclophosphamide	150	150	150	150	$S+G_2$	whole cycle
Vincristine	2				M	S
Ara-C	150	150	150	150	S (G_1/S)	S
Prednisone	50×4	50×4	50×4	50×4	G_1	G_1

cycle changes. On the other hand, peripheral blood is convenient for obtaining multiple samples and may be representative for the leukaemic population during the florid phase of leukaemia.

The present work has applied multiple drug regimens for the treatment of acute lymphatic and myeloid leukaemia (Table 1). FCM analysis of relative cellular DNA content in peripheral blood was done during therapy to determine whether a clinical or haematological response could be predicted, since FCM measures the cell cycle distribution at a high rate, and an accumulation of cells in a certain phase will rapidly be detected.

MATERIAL AND METHODS

The material consists of 12 patients of which 4 had acute lymphatic leukaemia (ALL) and 8 myeloid leukaemia (AML). Table 2 gives a survey of the haematological and clinical condition before and after the treatment cycles.

The treatment given for acute lymphatic leukaemia was COAP and for myeloid leukaemia PRAP (Table 1).

Blood samples were drawn each hour for 6 h, and thereafter each day during the treatment cycle. Monitoring of haemoglobin, leucocytes, thrombocytes, peripheral blood smears and FCM analysis was done on each sample. The separation of leucocytes for FCM analysis was: 5 ml blood was mixed with 0.5 ml Dextran 150 (100 g/l; Pharmacia, Sweden) and allowed to sediment for 20 min at 20° C (Talstad 1970). The leucocyte layer was pipetted off, and washed 3 times in phosphate buffered saline and fixed in 70 % alcohol. Before staining with ethidiumbromide, the cells were treated with pepsin and RNAse (Bersham 1972). The relative cellular DNA content was measured with Biophysic cytofluorograf 4802 A (Biophysics Instrument, Mahopac, New York), about 5×10^4 single cells per sample. Fractions of the samples with DNA content corresponding to the different cell cycle phases were estimated using a planigraphic method (Göhde 1973).

RESULTS

Haematological and clinical responses to the COAP or PRAP regimens (Table 2) were in most cases preceded by a pronounced increase in cells with relative DNA content corresponding to the DNA synthesis

TABLE 2

Clinical data before and after treatment – Definitions are given below

Patient	Age years/ sex	Peripheral blood leucocytes/µl		Blasts %		Bone marrow blasts %		Haematologic response	Clinical condition after treatment	FCM duration of initial S-phase increase (h)
		1[a]	2[b]	1[a]	2[b]	1[a]	2[b]			

TABLE 2

Clinical data before and after treatment – Definitions are given below

	Patient	Age years/ sex	Peripheral blood leucocytes/μl		Blasts %		Bone marrow blasts %		Haematologic response	Clinical condition after treatment	FCM duration of initial S-phase increase (h)
			1[a]	2[b]	1[a]	2[b]	1[a]	2[b]			
ALL	1. O.A.	18 ♂	3.1	0.8	5	0	96	5	+ + +	+ + +	4
	2. R.K.	16 ♂	6.8	0.5	60		77		+	+	6
	3. O.T. I	46 ♂	126	5.7	98	4	98	83	+ +	+ +	6
	3. O.T. III		7	1.2	80	80	90		−	+	
	4. G.H.	35 ♀	2.1	0.3	70	20	77	74	+	+	4
AML	5. T.G.	46 ♀	1.0	2.8	70	5	57	75	+ +	+	6
	6. G.G.	59 ♂	11	1.7			90	90	−	+	−
	7. A.H.	51 ♂	31	6.5	80	86	68	71	−	+	−
	8. A.P.	26 ♂	65	51	96	50	80	89	−	+	
	8. A.P. II		18.9	14.5	50	0	89	15	+ + +	+ +	3
	9. A.W.	67 ♂	5.9	3.5	34		49	34	+	+	6
	10. L.B.	72 ♂	115	18	85	85	20	38	+	+	5
	11. O.B.	16 ♂	7.1	3.8	58	32	95	88	+	0	
	12. V.A.	32 ♂	1.4	1.0	73	*	84	+ mors	*	0	

Haematopoietic response

−	No response:
+	Slight improvement:
+ +	Partial remission:
+ + +	Remission:
*	Died from leucopenia and septicaemia before assessment could be made

Percentage of blasts and promyelocytes

Peripheral blood	Bone marrow
High	High
Decrease	High
0 to 5	> 10
0	< 10

Clinical condition

0	Severely ill
+	Bad
+ +	Good
+ + +	No symptoms

a) Values before treatment
b) Values before next treatment

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phase of the cell cycle. This increase could be observed within half an hour and its mean duration was 5 h. However, there was no correlation between the magnitude and duration of the increase of 'S-phase' cells.

Typical FCM curves are shown in Figure 1. Calculated curves showing 'response' and 'nonresponse' are shown in Figure 2. Two patients (R.K. and A.W.) who showed initial S-phase changes died of septicaemia

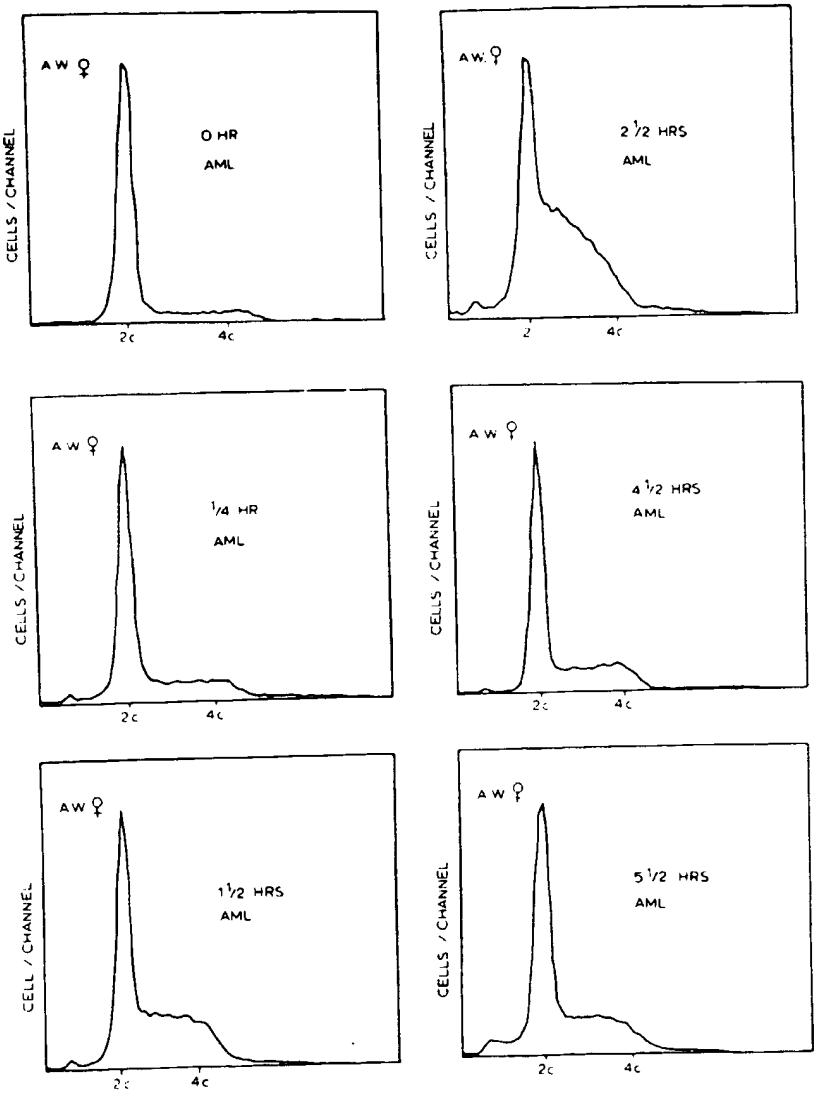
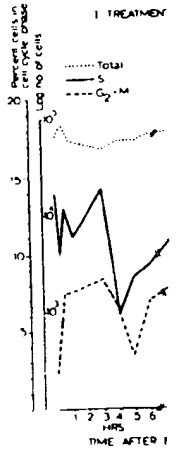
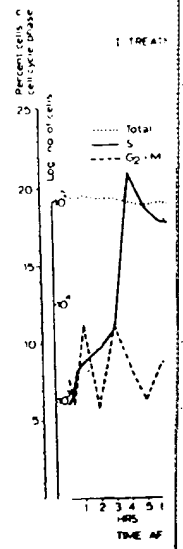


Figure 1. A typical FCM curve of peripheral blood leucocytes showing a rapid and prolonged increase of cells with S-phase DNA content. Patient with AML who showed haematological improvement.





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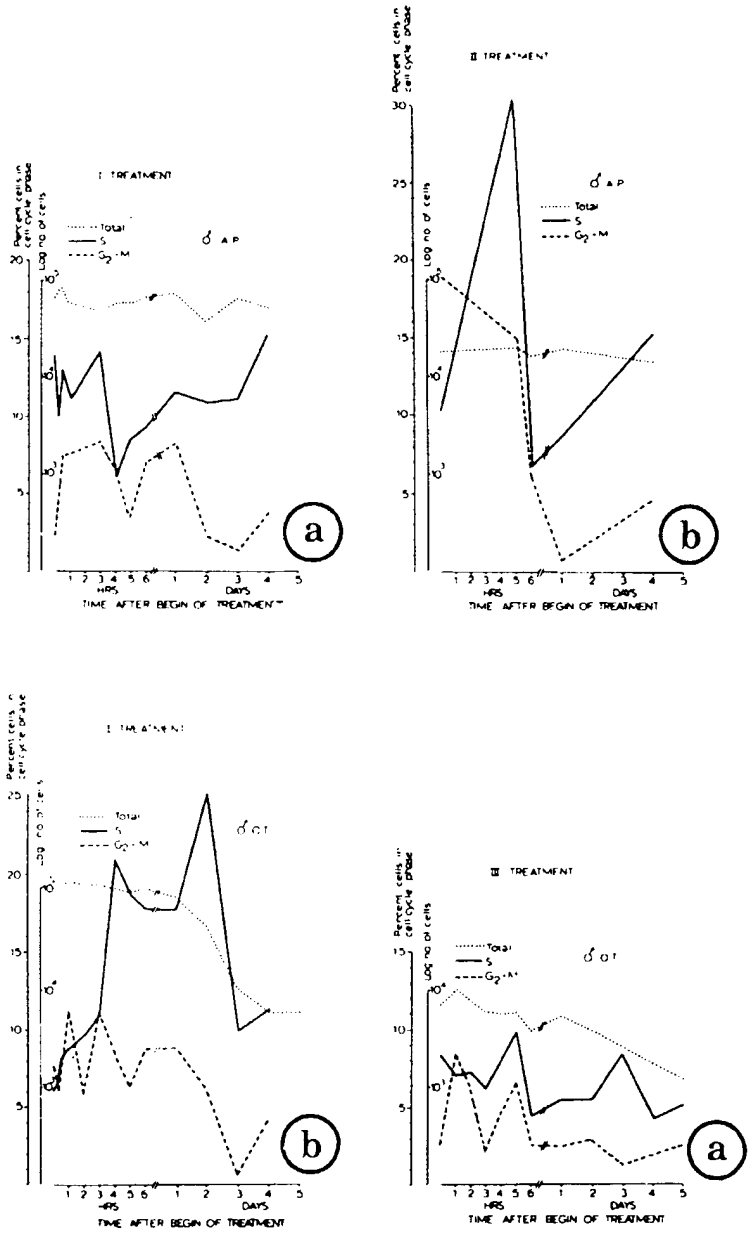

Figure 2. Calculated curves for relative DNA distribution corresponding to different cell cycle phases during therapy. PRAP

a. Patient not responding clinically to therapy.

b. Patient responding clinically to therapy.

before haematological or clinical response could be detected. At autopsy partial normalization of bone marrow was found in A.W. 1 patient (A.P.) who had no haematological or clinical improvement and no S-phase increase in the first treatment cycle, showed a marked S-phase increase and went into remission at the next cycle.

2 patients had an aneuploid DNA-peak. The FCM curves for one of them show the rapid disappearance of the aneuploid population during therapy (Figure 3). Similar curves were registered for the other patient, but both patients died with leucopenia and septicaemia before the cycle of treatment was completed.

DISCUSSION

Since most agents in the COAP or PRoAP regimens act on cells prior to or during the S-phase, one might except changes in the relative cell cycle distribution during treatment. However, the increase of cells with S-phase DNA content came more rapidly than might be expected, since the S-phase in leukaemia, lasts about 20 h (Gavosto & Pileri 1971). A possible explanation could be release of damaged S-phase cells from bone marrow or marginated pool. Such an increase in 1 patient who had only 5 % blasts in peripheral blood favors this explanation.

Another possible explanation is recruitment of resting cells into

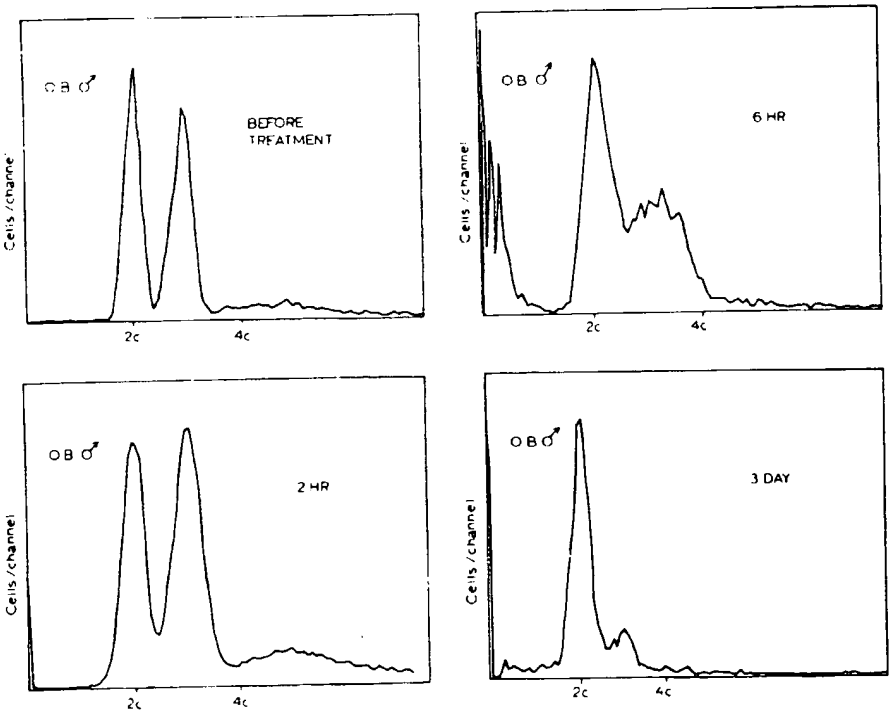

Figure 3. DNA distribution curves from a probable aneuploid leukaemia (AML) and sequence of reaction to treatment.

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They used ^{3}H-thymidine labelling for estimating S-phase, thus being in accordance with our FMF results from peripheral blood.

I[illegible]as earlier been shown by several workers that the reaction to cytostatic drugs may be visualized by FMF (Tobey & Crissmann 1972, Yataganas & Clarkson 1974, Krishan et al 1976, Barlogie et al 1976). In a recent study Smets (1976) found that clinical response to treatment could be predicted from the DNA-distribution in bone mar[illegible] cells 24 h after the start of the treatment.

Peripheral blood may not necessarily reflect the proliferative fraction of the leukaemic population in bone marrow. However, in the florid phase of leukaemia it might be as useful as bone marrow for detecting the cell cycle changes during treatment. It has seve[illegible] practical advantages. Sampling is conve[illegible]ient, the total leucocyte number can easily be registered and the cell cycle distribution can be followed sequentially.

As shown in one of our patients (A.P.) no response by FCM analysis at one treatment cycle did not exclude response at the next. A possible explanation is that adriamycin, which interferes with DNA synthesis, can accumulate in the body. After an initial fall following a single injection, the blood levels of adriamycin tend to be constant for about 7–10 d (Di Fronzo et al 1973). Possibly, at the next cycle of treatment 10 d after the first, the new dose of adriamycin was necessary to reach therapeutic levels for these particular cells.

If our results are reproducible on a larger scale, sequentical FCM analysis of peripheral blood may be of value for the early prediction of response to treatment.

ACKNOWLEDGEMENTS

This study was supported by the Norwegian Cancer Society. We thank Miss Gro Olderöy and Mrs. Signe Ögaard for valuable technical assistance.

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H. PFLIEGER,[1] R. ARNOI F. HAGHOU,[1] B.

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2) Gabriel H.H.W. et al., Overtraining and immune system: A prospective longitudinal study in endurance athletes.
Medicine and Science in Sports and Exercise, (1998) 30/7 (1151-1157).

3) Loppow D.et al., Flow cytometric analysis of the effect of dithiothreitol on leukocyted surface markers.
European Respiratory Journal, (2000) 16/2 (324-329).

4) Han K et al., Human basophils express CD22 without expression of CD19
CYTOMETRY, (1999 Nov 1) 37 (3) 178-83.

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JOURNAL OF RHEUMATOLOGY, (2000 Jun) 27 (6) 1341-51.

6) Girodon F. et al., Immunophenotype of a transient myeloproliferative disorder in a newborn with trisomy 21.
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SCANDINAVIAN JOURNAL OF HAEMATOLOGY, (1979 Jan) 22 (1) 25-32.

Thank you

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Overtraining and immune system: a prospective longitudinal study in endurance athletes

HOLGER H. W. GABRIEL, AXEL URHAUSEN, GÜNTER VALET, UTE HEIDELBACH, and WILFRIED KINDERMANN

Institute for Sports and Preventive Medicine, Faculty of Medicine, University of the Saarland, Saarbrücken, GERMANY; and Max-Planck-Institute for Biochemistry, Martinsried/Munich, GERMANY

ABSTRACT

GABRIEL, H. H. W., A. URHAUSEN, G. VALET, U. HEIDELBACH, and W. KINDERMANN. Overtraining and immune system: a prospective longitudinal study in endurance athletes. *Med. Sci. Sports Exerc.*, Vol. 30, No. 7, pp. 1151–1157, 1998. A prospective longitudinal study investigated for 19 ± 3 months whether immunophenotypes of peripheral leukocytes were altered in periods of severe training. Leukocyte membrane antigens (CD3, CD4, CD8, CD14, CD16, CD19, CD45, CD45RO, and CD56) of endurance athletes were immunophenotyped (dual-color flow cytometry) and list mode data analyzed by a self-learning classification system in a state of an overtraining syndrome (OT; $N = 15$) and several occasions without symptoms of staleness (NS; $N = 70$). Neither at physical rest nor after a short-term highly intensive cycle ergometer exercise session at 110% of the individual anaerobic threshold did cell counts of neutrophils, T, B, and natural killer cells differ between OT and NS. Eosinophils were lower during OT, activated T cells ($CD3^+HLA\text{-}DR^+$) showed slight increases (NS: 5.5 ± 2.7; OT 7.3 ± 2.4% $CD3^+$ of cells; means ± SD; $P < 0.01$) during OT without reaching pathological ranges. The cell-surface expression of CD45RO ($P < 0.001$) on T cells, but not cell concentrations of $CD45RO^+$ T cells, were higher during OT. OT could be classified with high specificities (92%) and sensitivities (93%). It is concluded that OT does not lead to clinically relevant alterations of immunophenotypes in peripheral blood and especially that an immunosuppressive effect cannot be detected. Immunophenotyping may provide help with the diagnosis of OT in future, but the diagnostic approach presented here requires improvements before use in sports medical practice is enabled. **Key Words:** STALENESS, IMMUNOPHENOTYPES, CD45RO, LYMPHOCYTES, FLOW CYTOMETRY, DIAGNOSIS, EXERCISE, ENDURANCE TRAINING

The leukocytosis of exercise has been known since the end of the last century (32). By investigating immune cells in peripheral blood, the only cell line that with some certainty to be impaired after strenuous exercise are the neutrophils (34). Reports about reduced cytotoxicity of natural killer (NK) cells, impaired *in vitro* proliferative responses of T and B lymphocytes, and altered functions of the monocyte/macrophage system are contradictory (26,29,33). Furthermore, so far the clinical relevance in healthy individuals of the measured effects in peripheral blood cells have not yet been uncovered. The difference between *in vitro* effects and clinically detectable phenotype contrasts with epidemiological findings. These findings show increased incidences of self-reported symptoms of upper respiratory tract infections (URTI) after strenuous endurance exercise (11,25,30). Also, personal experiences of athletes, coaches, and team physicians after single bouts of exercise under extremely hard conditions and or during periods with high training loads and/or increased frequency of competitions, especially if other stressors (psychological distress, malnutrition, weight loss, drugs, and disturbance of biological rhythms) coincide, suggest that incidence of infections—especially URTI—is higher than in other training periods (4). These experiences contrast with results of studies about overtraining/overtraining syndrome that cannot prove the hypothesis of increased URTI (5,15,19,21,22,28,31,43).

The present prospective longitudinal study investigated the impact of the overtraining syndrome on immune (presented here) and other (40,41) parameters. Cell line–specific and function-related surface receptors were measured to find parameters for diagnostic purposes. Common laboratory methods (immunophenotyping and flow cytometry) combined with a new self-learning diagnosis system served to help with the diagnosis of an overtraining syndrome.

MATERIALS AND METHODS

Study Design

Approximately 3–5 months apart, each individual (12 cyclists, 3 triathletes; age: 23.4 ± 6.7 yr, height 178 ± 7 cm, body mass 68.9 ± 7.0 kg, body fat 12.5 ± 2.1%, heart volume 14.0 ± 1.7 $mL \cdot kg^{-1}$, $\dot{V}O_{2max}$ 61.2 ± 7.5 $mL \cdot min \cdot kg^{-1}$) was investigated five times. Each of these investigations consisted of standardized tests over 2 separate days. The total time of the study was 19 ± 3 months. In

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TABLE 1. Absolute cell counts of leukocyte and lymphocyte subpopulations.

	NS	OT
Leukocytes	5131 ± 811	4954 ± 735
Neutrophils	3101 ± 788	2993 ± 770
Eosinophils	278 ± 176	211 ± 176*
Monocytes	345 ± 101	360 ± 150
Lymphocytes	1499 ± 342	1479 ± 414
$CD4^+CD45RO^-$	293 ± 132	278 ± 116
$CD4^+CD45RO^+$	319 ± 121	306 ± 102
$CD8^+CD45RO^-$	312 ± 123	286 ± 110
$CD8^+CD45RO^+$	155 ± 82	177 ± 91

*$P < 0.001$ compared with NS.
Means and SD; NS: normal status; OT: overtraining syndrome.

agreement with the individual training and competition program of each athlete, a period opportune for induction of OT was chosen, although the procedure of induction was not strictly defined. All investigations were performed on the same time of the day after an overnight fast. Before laboratory testings, training sessions were recorded for 2 wk, and most of the training sessions were monitored for heart rates. On the day before each testing, only regenerative training sessions were allowed. The last intensive or longer lasting training was at least 36 h before testing. Each individual gave informed written consent before the start of the study, which was approved by the Faculty of Medicine of the University of the Saarland.

The first day of each investigation comprised the following tests: present clinical and training history, physical examination, anthropometric measurements (45), resting ECG, incremental graded spiroergometry with ECG, and indirect measurement of blood pressure. In addition, on the first day of the first investigation, heart volume was measured by combined one- and two-dimensional echocardiography (modified Simpson rule (2); Vingmed CFM 700, Sonotron Inc., Norway). On the second day of each investigation, 3–7 d later, present history was taken again, a standardized psychological questionnaire was filled in and a highly intensive short-endurance exercise to exhaustion ("stress test") was performed to take repeated blood samples for determination of immunological parameters.

Two experienced physicians independently diagnosed OT by exclusion of other reasons, e.g., organic diseases. Classical symptoms as decrease of performance (reduction of results at recent competitions, unexpectedly premature interruption of training or competition), decreased subjective performance capacity and early fatigue with training going along with more or less severe vegetative symptoms (13,14,17,20). At the time of diagnosis, no subject suffered from infectious disease or diminished iron stores, determined by clinical examination and routine laboratory parameters.

Ergometry

All exercises were performed on electrically braked cycle ergometers in the upright position. An incremental graded exercise was conducted to subjective exhaustion as described in detail before (6,12,35).

The stress test consisted of an endurance exercise 10% above the maximal lactate steady state performed to subjective exhaustion (6,39). In 10-min intervals during the stress test, the athletes estimated their subjective rating of exertion using the Borg scale (1). At least 15 min after insertion of an catheter into an antecubital vein and after 15 min of quiet supine rest, the first blood sample was taken, the second at the end of the 10th min of exercise, and the third and fourth immediately and 1 h after exercise, respectively.

Immunophenotypes

One- and two-color indirect immunofluorescence technique was used to determine leukocyte and lymphocyte subpopulations in whole blood as described in detail before (42). Cell concentrations were corrected for plasma volume changes (3). Linear FSC and SSC scatter signals in combination with four-decade logarithmic FITC and PE fluorescence signals of lymphocytes, monocytes, and granulocytes were collected with a FACScan flow cytometer (BD) and were stored as FCS1.0 list mode files (6). The detailed procedures of processing the FCS1.0 list mode data are provided in Valet et al. (42), and CLASSIF1 program system (Partec, Münster, Germany) was used for calculations.

Statistics

Data are shown as means and standard deviations. Medians were calculated for each individual in case of not-overtrained normal state (NS). Statistical comparisons between NS and OT were made using the Wilcoxon test for matched pairs. The level of the significance was set at 2.5% ($P < 0.025$).

RESULTS

General Aspects and Performance

In 15 of 85 examinations, OT was diagnosed. Within these 15 OT cases, 6 appeared during the competition pe-

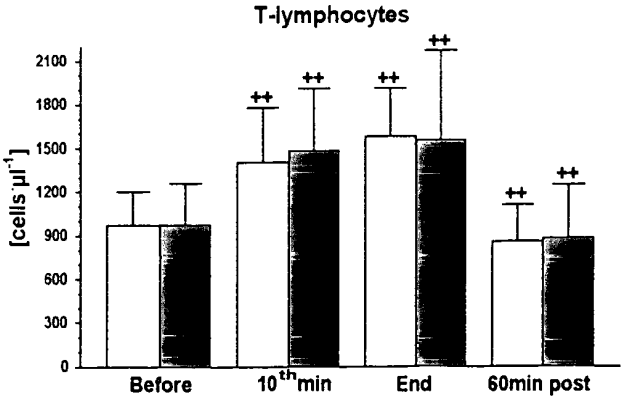

Figure 1—T lymphocyte ($CD3^+$) counts in overtraining (OT; *black bars*) and normal conditions (NS; *open bars*) of endurance athletes before, at the end of the 10th minute, at the end, and 60 min after a highly intensive endurance exercise to volitional exhaustion at 110% of the individual anaerobic threshold (OT: 16 ± 6 min; NS: 23 ± 10 min). Means ± SD; + $P < 0.025$, ++ $P < 0.01$ in comparison to values before exercise; * $P < 0.025$, ** $P < 0.01$, *** $P < 0.001$ between NS and OT; $N = 15$.

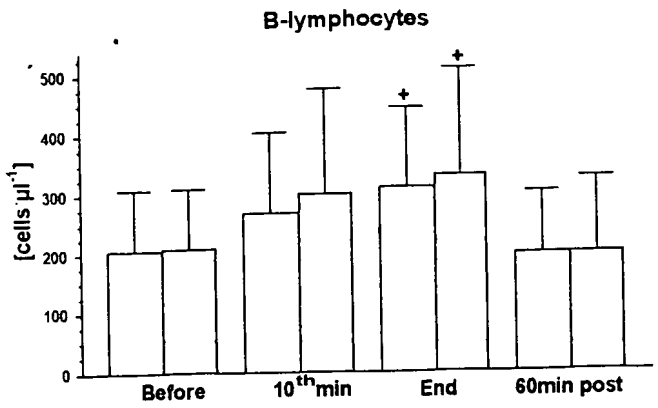

Figure 2—B lymphocyte (CD19$^+$) counts. For further information see Figure 1.

riod. OT was experimentally induced in 12 cases, mostly by a substantial increase of high-intensive training during 2–3 wk without the usual regenerative days, or by prolonging the competition period or a training camp. Weekly training volume before OT was not different from NS (approximately 10 (OT) and 9 (NS) h). The athletes had significantly increased their amount of training at an intensity above or within the range of the individual anaerobic threshold before OT (approximately 4.5 h per week) in comparison to NS (approximately 1.5 h per week). The athletes complained about typical OT symptoms: the feeling of heavy muscles of the lower limbs at modest exercise intensities, 13 athletes complained about intense daily fatigue and lack of concentration, 11 reported sleeping disorders, 4 a diminished appetite, and 3 an increased sweating rate. These complaints had started 13 ± 4 d before the examination date and lasted for 24 ± 10 d.

Borg-scale values were significantly higher after 10 min of the stress test during OT (OT 16.3 ± 1.5; NS: 14.3 ± 1.3; $P < 0.01$) without differences to NS at the end of this exercise test (all ratings >18). The following factors of the self-condition scale according to Nitsch (27) were altered significantly during OT: mean of all 14 binary factors as a measure for the global mood profile, fatigue, recovery, strain, sleepiness, and satisfaction (41).

Time to exhaustion of the stress test was significantly less by 27% during OT (16 ± 6 min) in comparison to NS (23 ± 10 min; $P < 0.01$). The maximal lactate concentration in the incremental graded exercise test was significantly decreased during OT (7.5 ± 2.7 mmol·L^{-1}; NS: 9.1 ± 2.4 mmol·L^{-1}; $P < 0.01$) (40,41).

Symptoms of URTI

Five of 15 athletes (33%) reported URTI symptoms during the 4 wk before the investigation dates. No athlete complained about severe generalized symptoms like chills or fever, but symptoms were localized to the URT (sore throat, rhinitis with clear secretion, mucosal swelling of the nose) in all but one case. One athlete reported a productive cough for a few days. Before the 70 investigations without exhibition of OT on 17 occasions, athletes reported URTI symptoms (24%). Three cases with fever and predominant symptoms of the URT were observed. During the last 2 wk before the investigations, severe infections were not recorded.

Immunophenotyp s: Cell Counts

Neither percentages nor absolute cell counts of the major cell lines (neutrophils, monocytes, B, total T, $T_{helper/inducer}$, $T_{suppressor/cytotoxic}$, and NK cells) showed differences before, during, or 60 min after the stress test. Particularly, the exercise induced mobilization of cells undergoing greatest fluctuations were not different (Table 1, Figs. 1-3). Among leukocyte subpopulations, eosinophils were lower during OT. HLA-DR$^+$ T cells (activated T cells) were slightly, but significantly, increased during OT (Fig. 4). Also, the percentage of CD16/CD56$^+$ among T cells was higher (Fig. 5). Cell counts of CD45RO$^+$ T cells, either CD4$^+$ or CD8$^+$, were not different between NS and OT (Table 1).

Immununophenotypes: Surface Antigen Contents

Among all surface antigens only CD45RO on both CD4$^+$ and CD8$^+$ T cells were higher during OT (Fig. 6). HLA-DR expression on T cells tended to be lower during OT, but differences to NS were not significant ($P = 0.089$). All other surface receptors did not show differences between OT and NS (Table 2).

Diagnosis of OT

The relative antigen content of CD45RO on both CD4$^+$ and CD8$^+$ T cells was the decisive data column to classify OT successfully. All other parameters were eliminated during repetitive iterations of the learning procedure. By using the 10th and 90th percentiles of normal samples, diagnosis of NS was correct in 84.3% and OT was recognized in 66.7% (Table 3, classification 1). Negative and positive predictive values were 96% and 44%, respectively. Subsequently, it was hypothesized that an increased expression of CD45RO indicates OT. A second classification tested how far the "overexpression" of CD45RO could recognize OT. The normal expression was associated with NS in 93.2% of

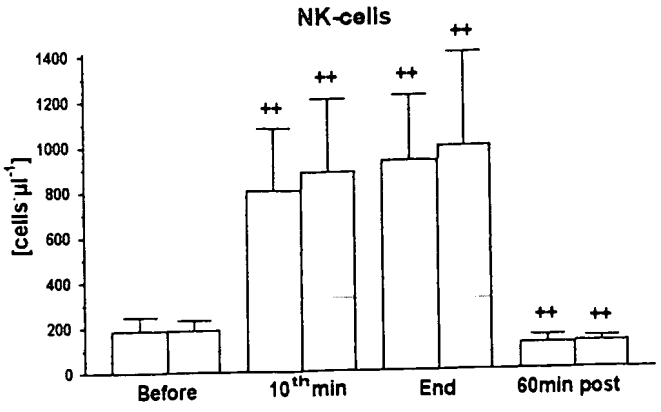

Figure 3—NK-cell (CD3$^-$CD16/CD56$^+$) counts. For further information see Figure 1.

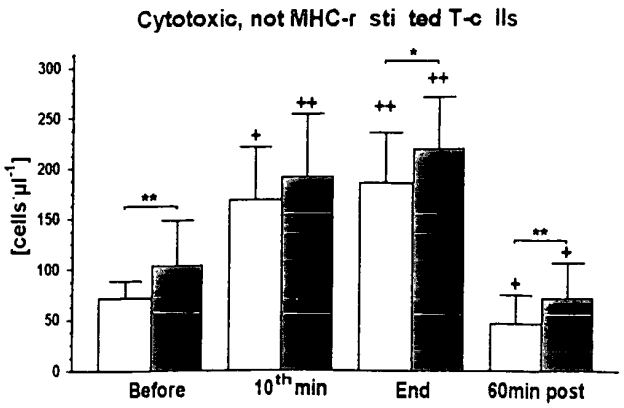

Figure 4—Cytotoxic, non-MHC-restricted T-cell counts ($CD3^+CD16/CD56^+$). For further information see Figure 1.

the cases and increased expression with OT in 93.3% (Table 3, classification 2; negative/positive predictive value: 96/67%). The third independent and prospective approach classified 19 unknown samples, which were independent from those of the study were classified. Eleven samples were from healthy athletes who were not overtrained. The other eight athletes suffered from an overtraining syndrome (clinical diagnosis). Ten of 11 normal samples and 5 of 8 OT samples were diagnosed correctly (Table 3, classification 3; negative/positive predictive values: 77/86%).

DISCUSSION

On the one hand, the present study provides information about unaltered distributions of all major cell lines. Only eosinophils showed reduced, activated T cells ($CD3^+HLA-DR^+$); and cytotoxic, non-MHC-restricted T cells ($CD3^+CD16/CD56^+$) moderately increased cell counts. On the other hand, new diagnostic aspects of OT by analyzing flow cytometrically achieved list mode data from immunophenotypes by using a new self-learning classification system were shown, although this classification procedure requires improvements for use in sports medical practice.

Contradicting results exist about total leukocyte counts during OT. Lehmann et al. (19) found reduced cell counts in eight overloaded middle/long distance runners, and Fry et al. (5) could not show altered leukocyte concentrations in five overtrained elite soldiers. Matvienko (23) presented reduced cell counts for athletes with stagnating performance, but without OT symptoms. On the basis of the present results, a reduction of the total leukocyte concentration is improbable. Also, in the here presented study, cell counts of all major cell lines detectable in peripheral blood, namely neutrophils, monocytes, B, total T, $T_{helper/inducer}$, $T_{suppressor/cytotoxic}$, and NK cells, were not altered during OT. In this context, the standardization of blood sampling conditions and laboratory methods are particularly important. The reason for reduced cell concentrations of eosinophils remains unclear. The mean eosinophil count of the five overtrained athletes reported by Fry et al. (5) was lower before starting the intensified training program. The immunological function of eosinophils are chemotaxis, adherence, phagocytosis, degranulation, production of lipid mediators, and reactive oxygen species (10). Activation of eosinophils leads to an increased production of cells in the bone marrow (e.g., in bronchial asthma) and induces an enhanced migration into inflamed tissues. Perhaps the phenomenon of a reduced eosinophil count during OT could be interpreted as an increased migration out of circulation but must remain without substantial proof at present.

The unaltered NK-cell counts presented here contradict literature findings. Fry et al. (5) reported about a decrease of NK cell concentrations under resting conditions during OT. This effect was likely due to relative high levels at the beginning of the study of one or two of the five subjects under investigation, if the high standard deviation is considered (mean 600, SEM 60 cell·μL^{-1}), and it remains questionable why. Such values are suspicious to have pathological or methodological reasons. It therefore is assumed, that no valuable effect on NK-cell numbers could be shown. In addition NK cells were determined as $CD56^+$ cells by using a single color immunofluorescence technique, which may have considerable overlap with $CD3^+CD56^+$ T cells and exclude $CD3^-CD56^-CD16^+$ NK cells. This and other papers presenting data about immunophenotypes in training studies showed further weak points from a methodological point of view. Single-marker studies (5,31,43) or incomplete description (15) of the use of marker combinations for T cells and T-cell subpopulations, and membrane activation markers such as HLA-DR, make it most difficult to interpret the results correctly. The CD4/CD8 ratio is easily influenced by $CD8^+$ NK cells, if single marker studies are used, and therefore does not necessarily represent a "helper/suppressor T-cell ratio" (15,43). Furthermore, there is no substantial reason to believe that this ratio plays "an important role in immunosurveillance" and a "ratio" below 1.5 being indicative of an increased susceptibility to infection (15,16). Cell separation methods, immunophenotyping protocols, standardized measurements, combination of monoclonal antibodies, and list mode data analysis were not directly comparable in many of the investigations (9) but have significant influence on the proportion of the one to the other cell subset.

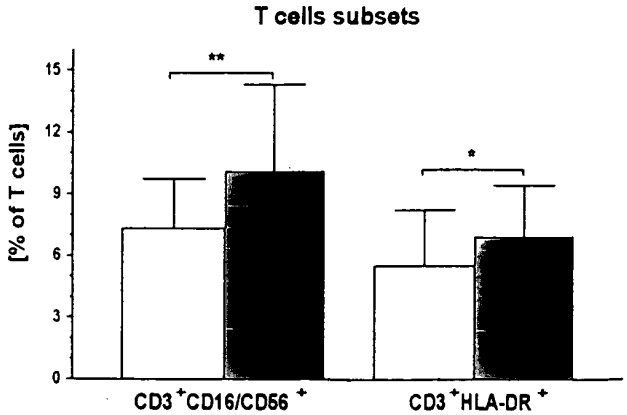

Figure 5—Cell counts of $HLA-DR^+$ or $CD16/CD56^+$ cells in percentage of $CD3^+$ T cells. For further information see Figure 1.

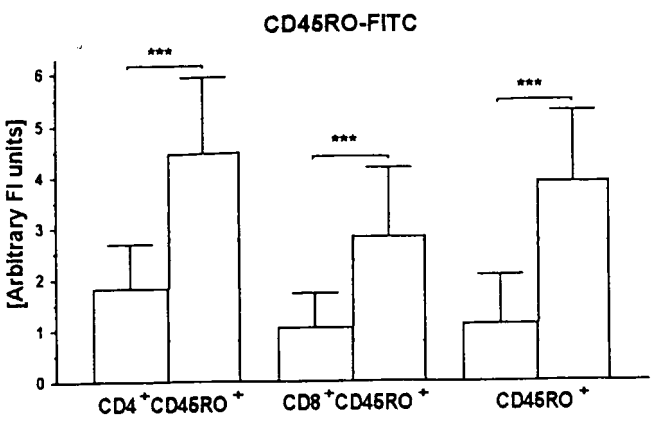

Figure 6—Mean fluorescence intensity (FI) of anti CD45RO-FITC on $CD4^+$, $CD8^+$, and total T cells ($CD3^+$). For further information see Figure 1.

If results of overtraining studies are regarded together with the present results, it can almost be excluded that overload training periods and an overtraining syndrome of relatively short duration leads to significant alterations of the distribution of the important immune cell lines detectable in peripheral blood. Cell concentrations of all major cell lines were unaltered during OT at physical rest. Furthermore, the mobilization into circulation and recirculation patterns after acute bouts of exercise during overtraining does not seem to be affected. This might be interpreted as an unimpaired trafficking of immune cells through the organism.

If the small increases of percentages of activated T cells expressing HLA-DR or interleukin-2 receptor (5) and unchanged HLA-DR expression on total lymphocytes (B cells + T cells) are regarded, the effect of intensified training and overtraining under the investigated conditions on the T cells must be regarded as marginal, especially if compared with activations that are induced by infections or other inflammatory processes in the organism. This is confirmed by studies of Verde et al. (43) showing trends of increased phytohemagglutinin- and concanavalin-stimulated lymphocyte proliferation, whereas others found a slightly impaired mitogenic-stimulated lymphocyte proliferation in four of five subjects (5).

The relatively high upregulation of CD45RO on T cells indicated a change in T-cell function, but it seems unlikely that this effect was specific for an OT. CD45RO is an isotype of the transmembranous tyrosine phosphatase CD45 (leukocyte common antigen (36)) and has its task with the T-cell receptor-mediated activation of lymphocytes (37,44). After immunogenic stimulus, T cells proliferate and express CD45RO instead of CD45RA, which is regarded as a late sign of activation (24). The intermediate population from $CD45RA^+$ to $CD45RO^+$ cells are $CD45RA^+CD45RO^+$ and expresses more interleukin-2 receptors than "naïve" $CD45RA^+$ T cells (46). One week after an 12-h duration endurance competition, this intermediate population is increased by 104% and indicates a moderate activation of T cells (7). Furthermore, the percentage of $CD45RO^+$ cells within total T cells increase with age (8). Viral infections, e.g., with Epstein-Barr virus, lead to a great increase of $HLA\text{-}DR^+$ and $CD45RO^+$ and $CD8^+$ (suppressive) T cells (18,38). The results of the present study show on the one hand an upregulation of CD45RO, but on the other hand a stable percentage of $CD45RO^+$ T cells, either $CD4^+$ or $CD8^+$. Obviously, a slight activation of T cells takes place but is not strong enough to increase the pool of circulating $CD45RO^+$ T cells. This effect considered together with the small increase of $HLA\text{-}DR^+$ T cells indicate a fine upregulation of the T-cell function, which at present is not seen as clinically relevant. On the other hand, an influence of URTI during the week before is unlikely, because seven athletes reported symptoms like sore throat or rhinitis, but this group did not show different expression densities for CD45RO or percentages of $HLA\text{-}DR^+$ T cells compared with those who did not report infectious symptoms ($N = 8$) during periods before OT. A further aspect might be that none of the athletes showed a high percentage of activated T cells or T cells expressing HLA-DR at high levels. This indicates that OT does not lead to a pathological enhancement of the T-cell function and the stimulus "OT" is only minimally immunogenic. Furthermore, it remains unclear which is the concrete immunogenic stimulus.

Although the immunologic meaning of the upregulated expression of CD45RO seems to be of minor importance, this upregulation enabled a differentiation between NS and OT. It was the first successful attempt of an diagnosis by using a self-learning classification on the basis of lymphocyte immunophenotypes (42). Percentages of immune cell lines of subpopulations determined by staining the activation receptors HLA-DR and CD45RO did not contribute to the diagnosis of OT. In general and beyond the aims of this specific hypothesis of the study, it must strongly be recommended to look at surface membrane contents (receptor densities) in addition to percentages and absolute cell counts of immune cell populations to achieve optimal results for clinical diagnoses. The present classification based on flow cytometric list mode data indicates that the clinical diagnosis of an OT could be confirmed with a sensitivity of about 67% (specificity: 84%). It seems speculative to regard an upregulation of CD45RO as a criterion for OT, but under the

TABLE 2. Membrane antigen contents in arbitrary fluorescence intensity units.

mAb-fluorescent dye	Lymphocyte subpopulation	Fluorescence intensity (arbitrary units)	
		NS	OT
antiCD3-FITC	$CD3^+$	2.26 ± 0.57	2.39 ± 0.64
antiCD19-FITC	$CD19^+$	1.05 ± 0.26	1.19 ± 0.20
antiCD16-PE and	$CD3^-CD16/CD56^+$	2.32 ± 1.12*	2.50 ± 1.04*
antiCD56-PE	$CD3^+CD16/CD56^+$	0.63 ± 0.29	0.65 ± 0.17
antiCD4-PE	$CD4^+CD45RO^-$	10.16 ± 3.19	10.16 ± 2.90
	$CD4^+CD45RO^+$	12.26 ± 3.62	11.74 ± 2.70
antiCD8-PE	$CD8^+CD45RO^-$	14.40 ± 3.75	14.11 ± 3.95
	$CD8^+CD45RO^+$	14.77 ± 4.37	15.02 ± 4.56
antiHLA-DR	$CD3^-HLA\text{-}DR^+$	5.35 ± 2.75*	4.68 ± 2.50*
	$CD3^+HLA\text{-}DR^+$	1.67 ± 1.40	1.32 ± 0.77
IgG-FITC	lymphocytes	0.05 ± 0.02	0.05 ± 0.01
IgG-PE	lymphocytes	0.05 ± 0.01	0.03 ± 0.01

*: $P < 0.001$ in comparison to value directly below.
†$P < 0.001$ in comparison to corresponding value at NS.
Means and SD; FITC: fluorescein isothiocyanate; PE: phycoerythrin; mAb: monoclonal antibody; NS: normal status; OT: overtraining syndrome.

TABLE 3. Confusion matrices for flow cytometric classification and clinical diagnosis of normal status (NS) and overtraining syndrome (OT).

		Flow Cytometrical Classification	
		NS	OT
Classification 1 Clinical diagnosis	NS (N = 51)	84.3	25.5
	OT (N = 15)	13.3	66.7
Classification 2 "Overexpression" of CD45RO	NS (N = 51)	92.2	13.7
	OT (N = 15)	13.3	93.3
Classification 3 Clinical diagnosis	NS (N = 11)	90.9	9.1
	OT (N = 8)	37.5	62.5

Classification 1: original data set; classification 2: hypothesis: overexpression of CD45RO indicates OT; classification 3: prospective classification of 19 unknown samples.

hypothesis that this would be the case, the upregulation of CD45RO and the clinical diagnoses of NS or OT could be done with very high sensitivity and specificity. The prospective approach of 11 NS and 9 OT (Table 4, classification 3) is more important for the confirmation of the results. Three interpretations are possible why "only" about 60% of OT was recognized. Probably the receptor upregulation of CD45RO differs interindividually, which means that each subject might not react at the same extent than others. Furthermore, OT might not always go along with an upregulation of CD45RO. Finally, it must be taken into account that a gold standard to diagnose OT does not exist and the clinical diagnosis of an OT need not inevitably be correct, although the long personal experience of two independent physicians provides a high probability for a correct clinically based diagnosis.

The present study was conducted under the prediction to induce an overtraining syndrome in endurance athletes over a time period over about 1.5 yr. Before drawing conclusions from the results, some aspects have to be included as follows. The diagnosis of OT was subjective but took into account recommendations from the literature. A gold standard to diagnose an OT does not exist. Furthermore, it is impossible to standardize time schedule for training and competitions over 1.5 yr. Protocols of training inclusively monitoring of heart rates had to serve as estimates for the performed physical loads. Also, results and conclusions must be restricted to endurance athletes at present and cannot be extended to other athletes. Last but not least, immunophenotypes of leukocytes derived from peripheral blood provide only limited information about the actual balance of the immune system of the whole organism.

From the present study the following conclusions are drawn. OT does not lead to clinically relevant alterations of immunophenotypes in peripheral blood. On the one hand, the moderate activation of T cells as shown by slight increases of the percentage of HLA-DR$^+$ T cells and the upregulation of CD45RO on T cells indicate an enhanced functional state of T cells. On the other hand, pathological ranges are not achieved, which excludes a significant activation of this part of the immune system. Unaltered exercise-induced mobilization and redistribution patterns of leukocyte and lymphocyte subpopulations indicate an unchanged flexibility to transport immune cells through the blood from one site to another. In consideration of the present results and the few other studies about overtraining and immune functions effects on immunophenotypes, *in vitro* proliferation response of lymphocytes to mitogens, secretory immunoglobulins, and also plasma glutamine levels cannot serve as parameters explaining the experiences of coaches, physicians, and athletes themselves of an increased susceptibility to infections in overtraining periods. So far, anecdotal reports about an increased susceptibility to infections cannot be confirmed by overtraining studies published up to date. It might help as an explanation for this apparent contradiction that most studies did not investigate top athletes in situations particularly likely to induce an overtraining syndrome like psychological stress, malnutrition, and postinfectious periods. Experimentally induced overtraining might not necessarily reflect a comparable situation. Last but not least, immunophenotyping of lymphocytes provided help with the diagnosis of OT in this study and will probably support the diagnosis of OT in future. This may be seen as an innovative and promising part of the present study, which requires improvements before it can be used in the daily routine of the sports medical practice.

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Thank you

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Enhanced Neutrophilic Granulopoiesis in Rheumatoid Arthritis. Involvement of Neutrophils in Disease Progression

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ABSTRACT. ***Objective.*** To investigate enhanced granulopoiesis in bone marrow of patients with rheumatoid arthritis (RA), and the role of neutrophils in RA pathogenesis.

Methods. Aspirated bone marrow cells and peripheral blood leukocytes from patients with RA and non-RA patient controls were analyzed morphologically and by 2 color flow cytometry. Thirteen iliac bones (8 RA, 5 non-RA) were examined by light and transmission electron microscope (TEM).

Results. The percentage of CD15+CD16– cells (immature neutrophils) in RA bone marrow (64.3 ± 13.4%, mean ± SD) increased significantly compared to that of non-RA controls (43.2 ± 14.3%), whereas the fraction of CD15+CD16+ cells (mature neutrophils)was greatly decreased (RA 21.8 ± 10.1%; non-RA 38.1 ± 8.9%). The absolute number of CD15+CD16– cells also increased markedly in RA bone marrow. The ratio of immature cells to the total granulocytes (% CD15+CD16– to % CD15+) correlated with the Lansbury Index score ($R = 0.76$, $p < 0.0001$). TEM observations revealed that abundant immature neutrophils adhered closely to the trabeculae of the iliac bone. Margins of trabeculae were mostly irregular, especially in severe RA, and collagenous fibers frequently disappeared in those trabeculae with ragged margins.

Conclusion. In RA bone marrow, immature neutrophils (CD15+CD16–) were markedly increased in number; by contrast, no changes were found for mature cells. Augmented production of immature neutrophils (at the promyelocyte-to-myelocyte stage) might lead to the destruction of collagenous fibers in RA bone trabeculae, as revealed by TEM. Generalized bone destruction in RA might, at least in part, be caused by enhanced production of immature neutrophils. (J Rheumatol 2000;27:1341–51)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
BONE DESTRUCTION
GRANULOPOIESIS
BONE MARROW
NEUTROPHILS

Rheumatoid arthritis (RA), an inflammatory disease by pathology[1], and an autoimmune disease by cause, is characterized by synovial hyperplasia and destruction of articular structures[2-4]. Into the thickened synovium lymphocytes infiltrate massively, frequently forming solitary follicular aggregations. Abundant polymorphonuclear cells (PMN) are observed at the cartilage–pannus junction and in the synovial fluid. In and around the inflamed joints, PMN play a role in the pathogenesis of RA; they contain destructive enzymes in their granules and may generate reactive oxidants.

In most cases, however, the synovium and the joints are not the only structures affected: the lesions extend to the bone marrow adjacent to the inflamed joint, and hemopoietic activity is often distinctively affected in patients with RA. Several reports describe the high levels of cytokines and the abnormalities of the cells in RA bone marrow. Elevations of interleukin 6 (IL-6) and IL-8 have been detected[5], and unusual myeloid cells bearing an oncofetal surface marker have been recognized in the bone marrow[6-8]. These abnormal cells were detected not only in bone marrow adjacent to the inflamed joint but also in iliac bone marrow remote from the arthritic lesion. In the iliac bone marrow of patients with RA, the number of bone marrow cells was markedly increased and the percentage of myeloid lineage cells (promyelocyte–myelocytes) in the mononuclear cell (MNC) fraction was substantially higher than in healthy individuals[7,8]. Although these reports suggest that granulopoiesis was affected and strongly enhanced by RA disease, little is known about the relationship between the state of granulopoiesis and disease activity.

We undertook flow cytometric analysis to examine

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whether there are any detectable quantitative changes in granulopoiesis in the iliac bone marrow and in the peripheral blood (PB) of RA. As well, we performed a series of morphological examinations of iliac bones of patients with RA to detect destructive changes of bones to investigate any possible relationship of bone destruction and elevated granulopoiesis.

The ratio of immature myeloid lineage cells was found to be positively correlated with disease severity, and the destruction of iliac bone trabeculae by immature neutrophils was strongly implicated by morphological, although circumstantial, evidence. Altogether, these data suggest that immature neutrophils abundantly produced in RA bone marrow are apparently involved in the generalized destruction of bone trabeculae in patients with RA.

MATERIALS AND METHODS

Patients. All patients in this study were treated at the Department of Orthopedic Surgery, Naruko National Hospital. Twenty-five patients with RA (16 women, 9 men) who satisfied the American Rheumatism Association diagnostic criteria were studied. As controls, 12 patients who had no inflammatory diseases (6 women, 6 men) were examined. Among them, 6 patients had lumbar disc hernia and another 6 had traumata. The average age of patients with RA was 61.6 years (range 49–83) and that of controls 55.1 years (range 43–73). The duration of RA ranged between one and 38 years (mean 14.8). Disease severity was evaluated based on the Lansbury Index score, and the mean score of RA patients in this study was 69.0% (range 41–102%)[9]. Among 25 patients, 14 were medicated with steroids (prednisolone, 5–10.0 mg/day), 22 had been treated with nonsteroidal antiinflammatory drugs, and 20 with disease modifying drugs. Table 1 shows the patients' data. Iliac bone specimens were obtained from 8 patients with RA who needed iliac bone grafts for total hip replacement. Table 2 gives details of iliac bones subjected to morphological examinations, with tentative classification of disease stages (comprehensive classification by Lansbury score and morphological findings). As controls, 5 iliac bones were obtained from patients undergoing surgery for lumbar fixation. Informed consent for procedures was obtained from each individual, and permission to carry out the study was granted by the ethical committee of the Naruko National Hospital.

Monoclonal antibodies (Mab). Fluorescein isothiocyanate (FITC) conjugated monoclonal anti-CD15 (C3D-1) was purchased from Dako (Carpinteria, CA, USA). It recognizes an epitope involving the carbohydrate sequence 3-fucosyl-N-acetyllactosamine and a broad range of myeloid lineage cells, from myeloblasts to PMN. Phycoerythrin (PE) conjugated monoclonal anti-CD16 (Leu-11c) (Becton Dickinson, Franklin Lakes, NJ, USA) was purchased to recognize FcαRIII on neutrophils and natural killer cells.

CD15 is expressed in a broad range of myeloid lineage cells[7,8]; CD16 is a receptor for IgG (FcαRIII). Since FcαRIII is expressed only in mature neutrophils, the CD15+CD16– reaction identifies immature myeloid cells, whereas CD15+CD16+ identifies mature myeloid cells. Immature myeloid cells were confirmed by morphology as promyelocytes–myelocytes.

Cell preparation. Three milliliters of bone marrow blood was aspirated by needle puncture from the posterior superior iliac crest under local or lumbar anesthesia, and the same amount of venous blood was obtained simultaneously. The syringes were coated with 0.1 ml of heparin before use. These samples were stored at 4°C until use. All examinations were performed within 8 h. Samples were centrifuged at 800 g for 5 min. To remove red blood cells, samples were treated twice with hypotonic lysis solution [0.15 M ammonium chloride, 0.01 M potassium bicarbonate, 0.1 mM ethylenediaminetetraacetic acid (EDTA; Kanto Chemical, Tokyo, Japan)][10] at room temperature. Blood specimens (3 ml from each) were first incubated with 10 ml of the hypotonic lysis solution for 5 min. Immediately after, the cells were spun down (200 g for 5 min), resuspended in Hanks' balanced salt solution (HBSS; Research Institute of Microbial Disease, Osaka, Japan), and again incubated with lysis solution at room temperature for 3 min. Finally, the cells were washed in HBSS 3 times. Each cell suspension was adjusted at 10^7/ml in PBS containing 2% heat inactivated fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, UT, USA) and 0.1% NaN_3 (PBS–2% FCS). This final suspension contained not only MNC but also PMN.

Flow cytometric analysis. Aliquots (0.1 ml) of bone marrow cell suspensions (1×10^7/ml) or PB leukocyte suspensions (1×10^7/ml) were stained with FITC conjugated anti-CD15 Mab and PE conjugated anti-CD16 Mab for 45 min on ice. Then cells were washed 3 times in PBS–2% FCS. Analyses were performed on FACScan with a Lysys program (Becton Dickinson). Dead cells were gated out by additional staining with 7-amino actinomycin D (Sigma, St. Louis, MO)[11].

Morphological examination. Bone marrow cells and peripheral leukocytes were cytospun onto glass slides for 5 min at 100 g and stained with May-Grünwald Giemsa.

For light microscopy, iliac specimens were fixed overnight in 4% paraformaldehyde in 0.01 M PBS. After washing with PBS, the specimens were decalcified in 10% EDTA in PBS for 2 weeks at 4°C[12,13]. After decalcification, specimens were dehydrated in a graded series of ethanol, passed through xylene, and embedded in paraffin. The paraffin embedded specimens were sectioned at 5 μm thickness and stained with hematoxylin and eosin.

For transmission electron microscopic (TEM) examination, bone specimens from patients with RA were fixed overnight (2.5% glutaraldehyde and 2% paraformaldehyde in 0.01 M PBS). After the decalcifying process, the specimens were transfered and postfixed in 2% OsO_4 for 2 h, dehydrated in a

Table 1. Details of the patients.

	Control	RA
Number	12	25
Age, yrs (range)	55.1 (43–73)*	61.6 (49–83)*
Male/Female	6/6	9/16
Duration of disease, yrs (range)		14.8 (1–38)*
Drug administration, no. of patients		
NSAID		22
DMARD		20
Steroid (prednisolone)		14
Dose, mg (range)		5.77 (5–10.0)*
Other measurements		
Lansbury Index		69.0 (41–102)*
CRP, mg/dl		5.6 (1.5–14.2)*
ESR, mm/h		69.3 (18–103)*

*Mean (range).

Table 2. Patients subjected to ultrastructural examinations.

Tentative (Comprehensive) Classification	Patient	Lansbury Index
Severe RA	1	102
	2	93
	3	74
Less severe RA	4	76
	5	73
	6	62
	7	60
Mild RA	8	41

graded series of ethanol, passed through propylene oxide, and then embedded in EPON 812 (Taab Inc., Reading, UK). Semithin sections were stained with 0.5% toluidine blue, and electron microscopic sections with uranyl acetate and lead citrate.

Before postfixation with OsO_4, some of the decalcified bone specimens were incubated 2 h with the reactive solution (0.06% diaminobenzidine and 0.03% H_2O_2 in 0.1 M Tris HCl buffer, pH 7.6) to represent myeloperoxidase[14,15].

Statistical analysis. Statistical differences between patients and controls were examined by Mann-Whitney U test. Significance was accepted with $p < 0.05$. Pearson's correlation coefficient was calculated to evaluate the correlation of Lansbury Index score with the ratio of immature/total granulocytes. The significance of this coefficient was tested using Fisher's Z transformation.

RESULTS

Number of total nucleated cells. The number of bone marrow nucleated cells of patients with RA was significantly increased compared with control patients (> 3-fold increase; $p < 0.005$). Although less significant ($p < 0.05$), there was also a statistical difference in the number of total nucleated cells in the PB between RA patients and controls (Table 3).

Flow cytometric analysis of myeloid fraction. Figure 1 shows the typical pattern of bone marrow cells by flow cytometry with CD15 and CD16. Although we found no statistically significant difference between the average ratio of CD15+ cells in the bone marrow of RA and control patients, the absolute number of CD15+ cells increased significantly in RA bone marrow, indicating the enhanced granulopoiesis in RA bone marrow. The percentage of CD15+CD16– cells was considerably increased ($p < 0.01$) in the RA bone marrow compared with control patients. In contrast, the percentage of CD15+CD16+ cells in RA bone marrow was decreased reciprocally ($p < 0.005$) (Table 3). In terms of the absolute counts, the mean number of CD15+CD16– cells in the RA bone marrow was greater than in the control bone marrow ($p < 0.001$) (Figure 2). Since the bone marrow of the RA patients was hyperplastic (Table 3), the number of CD15+CD16+ cells in RA was found to be comparable to that of the control bone marrow, even though the percentage of CD15+CD16+ cells in RA bone marrow was markedly low (Figure 2).

Although there was no significant difference in the percentage of CD15+CD16+ cells in the PB, the average ratio of

Table 3. The subsets in bone marrow and peripheral blood cells. Cells were stained by 2 color staining. Analysis was performed with a FACScan, and the percentages of positive cells were determined using Lysys software. The number of cells in peripheral blood was measured with a Coulter counter. Data represent mean ± SD of observations.

Source	No. of Cells, 10^6/ml	Percentage Positive Cells CD15+	CD15+CD16–	CD15+CD16+
Bone marrow				
Control	3.4 ± 2.0	81.4 ± 6.6	43.2 ± 14.3	38.1 ± 8.9
RA	11.1 ± 6.3***	86.1 ± 6.8†	64.3 ± 13.4**	21.8 ± 10.1***
Peripheral blood				
Control	5.6 ± 1.1	80.9 ± 9.2	4.4 ± 2.2	76.4 ± 9.8
RA	7.6 ± 2.0*	83.9 ± 8.7†	17.7 ± 26.3†	66.5 ± 27.3†

†Not significant. *$p < 0.05$ vs control. **$p < 0.01$ vs control. ***$p < 0.005$ vs control.

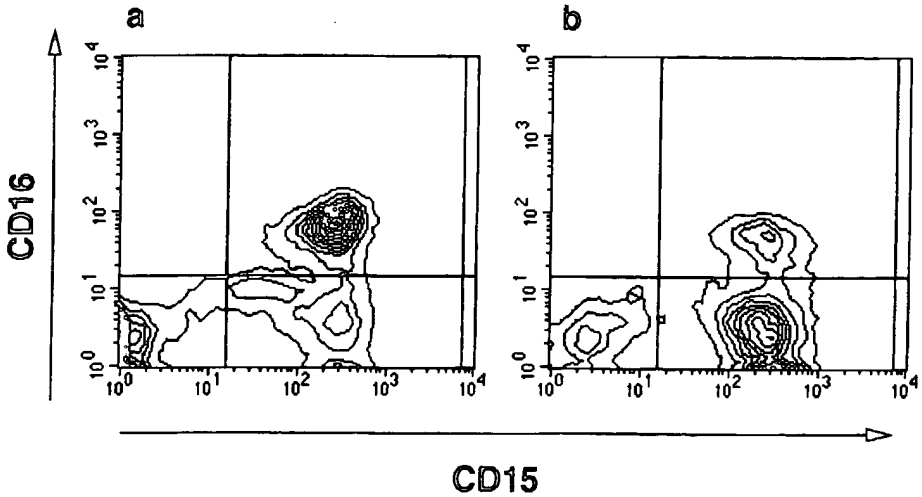

Figure 1. Typical flow cytometric profiles of iliac bone marrow cells from a control (OA) patient (a) and a patient with RA (b). 2 color FACS analysis of iliac bone marrow cells with anti-CD15 and anti-CD16. The ratio of CD15+CD16– cells (immature granulocytes) increased significantly.

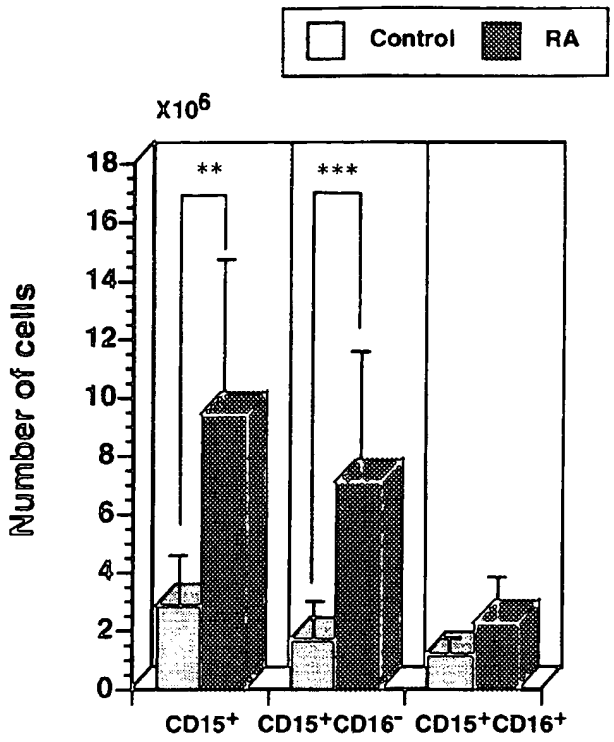

Figure 2. Mean numbers of CD15+, CD15+CD16–, and CD15+CD16+ cells in bone marrow of control and RA patients. **p < 0.005 compared to control; ***p < 0.001 compared to control.

CD15+CD16– cells was larger in the RA group than in the control group (Table 3), and was similar to the result obtained in the bone marrow.

Ratio of immature myeloid lineage cells correlated with the Lansbury Index. A possible relationship was investigated between the prominent population of immature myeloid cells in RA bone marrow and the clinical symptoms of patients with RA. The Lansbury Index was plotted against the ratio of immature granulocytes (percentage of CD15+CD16– cells) to whole granulocytes (immature plus mature granulocytes; percentage of CD15+ cells). The Lansbury Index score was found to be closely correlated with the ratio of immature granulocytes ($R = 0.766$, $p < 0.0001$) (Figure 3).

Morphological study: bone marrow and peripheral blood cells. The light microscopic observations with the cytospin preparation of bone marrow and PB by May-Grünwald Giemsa staining supported the findings from the flow cytometric study. In the RA bone marrow, immature myeloid cells, i.e., promyelocytes and myelocytes (of neutrophilic lineage) appeared to be dominant, and consequently hemopoietic cells of other lineages could rarely be detected (Figures 4C, 4D) compared to the control bone marrow, where, in addition to numerous mature cells of neutrophilic lineage, erythroid cells at various stages were frequently observed (Figures 4A, 4B).

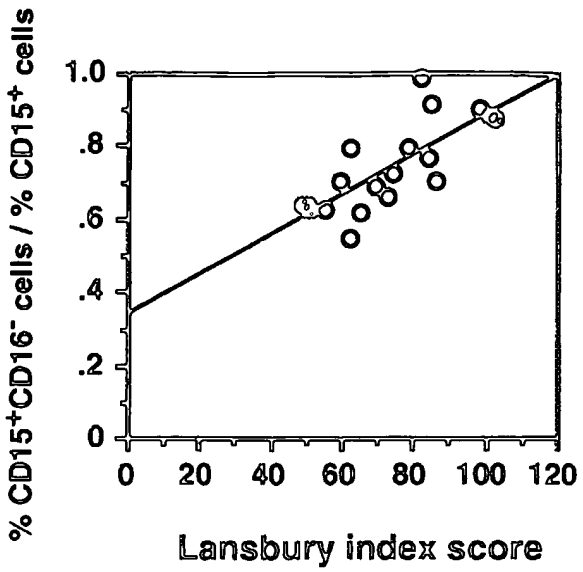

Figure 3. Correlation of the Lansbury Index score with the ratio of immature neutrophils [ratio of (percentage of CD15+CD16– cells) to (percentage of whole CD15+ cells)]. $R = 0.766$, $p < 0.0001$.

Morphological features similar to those found on bone marrow cells were observed in PB cytospin preparations of patients with RA. Nearly equal numbers of mature neutrophils (PMN) were observed in cytospin preparations from both RA and control groups (data not shown), as expected from Table 3. Remarkable and noteworthy were immature myeloid cells, such as promyelocytes and myelocytes, that were observed in the PB from 2 patients with severe RA (Figure 5).

Light microscopic observation of iliac bones. Control (osteoarthritis, OA) patients had smooth surfaced bone spicules. Lining cells could be readily observed on nearly all spicules (Figure 6A). In patients with severe RA, however, irregularly shaped trabeculae were frequently observed in sections stained with hematoxylin and eosin (Figure 6B). Lining cells could not be detected around these irregularly shaped trabeculae, in contrast to those of control patients. Numerous hemopoietic cells around the spicules were another striking feature in RA bone marrow (Figure 6B).

Smooth surfaced bone spicules with apparent lining cells were also confirmed in both semithin sections from OA control patients (Figure 7A) and from less severe RA (Figure 8A). A modest but significant number of immature myeloid cells could be found on the surface of the bone trabecula from the patients with less severe RA. Semithin sections of iliac bones of patients with severe RA, whose PB contained promyelocytes and myelocytes (Lansbury index 102), revealed irregular trabecular contours with adherent small cells, as well as a number of rather large nucleated cells (Figure 9A). The surfaces of these bones with ragged margins from patients with RA were mostly devoid of lining cells. Abundant hemopoiet-

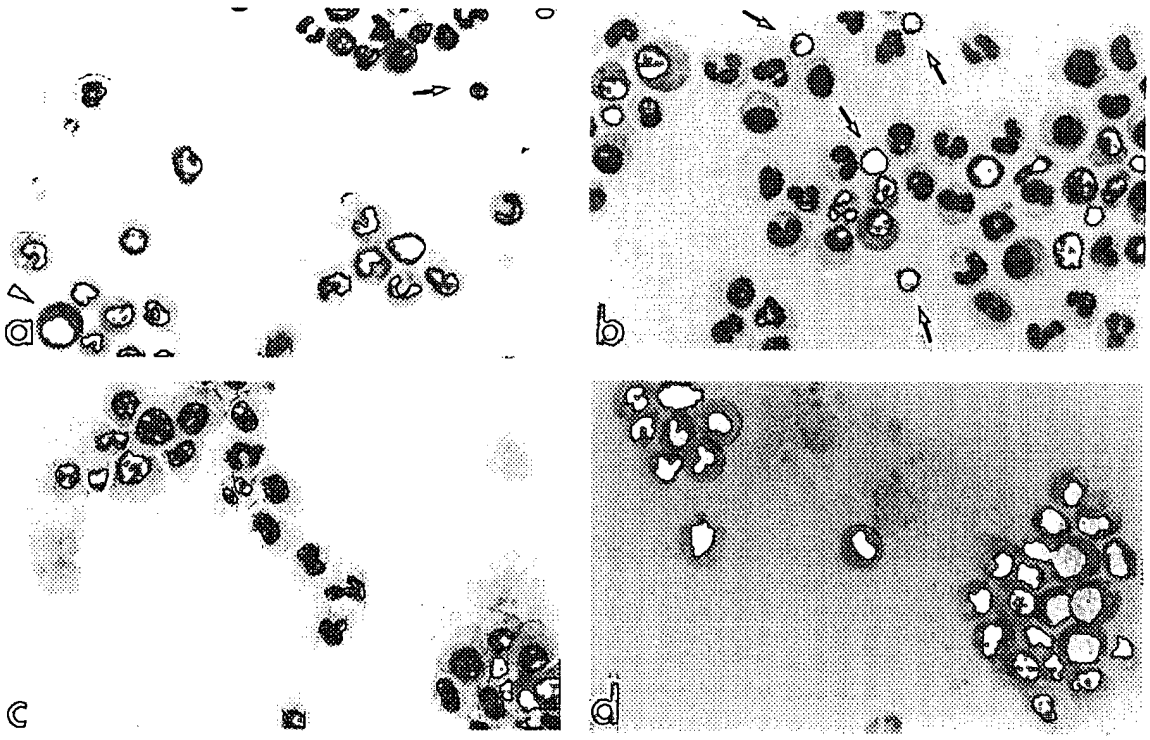

Figure 4. May-Grünwald Giemsa staining of bone marrow cells from a control (OA) (a, b) and a patient with RA (c, d). In control bone marrow, erythroid lineage cells were frequently observed in addition to myeloid lineage cells (a, b), while apparently immature myeloid cells were prominent, and hemopoietic cells in other lineages could hardly be seen in the RA bone marrow (c, d). Arrowhead indicates a proerythroblast, arrows normoblasts (a, b, ×900).

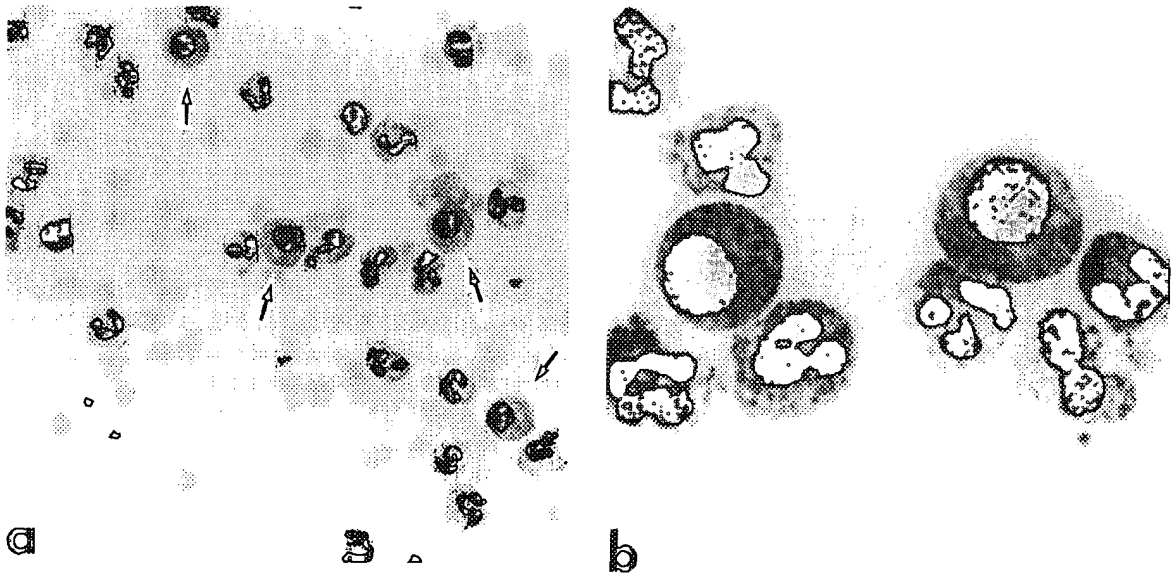

Figure 5. May-Grünwald Giemsa staining of peripheral leukocytes of a patient with severe RA (Lansbury Index 102). (a) Myeloid cells at a relatively immature stage (arrows) are frequently observed. (b) Higher magnification; immature myeloid cells can be identified as promyelocytes–myelocytes based on their characteristic morphology (a ×900, b ×2200).

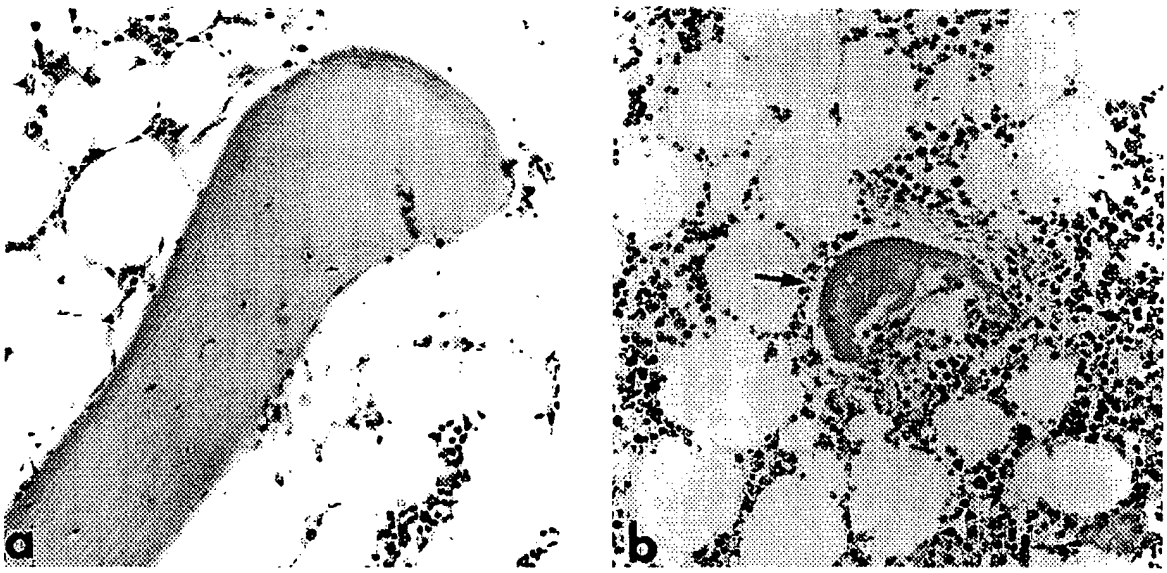

Figure 6. Micrographs of the iliac bone of a control (OA) patient (a) and a patient with severe RA (b) stained with H&E. An irregularly shaped trabecula can be seen in the RA bone (arrow), while the control trabecula has a smooth surface. Hematopoietic cellularity in the bone marrow is augmented in RA (×220).

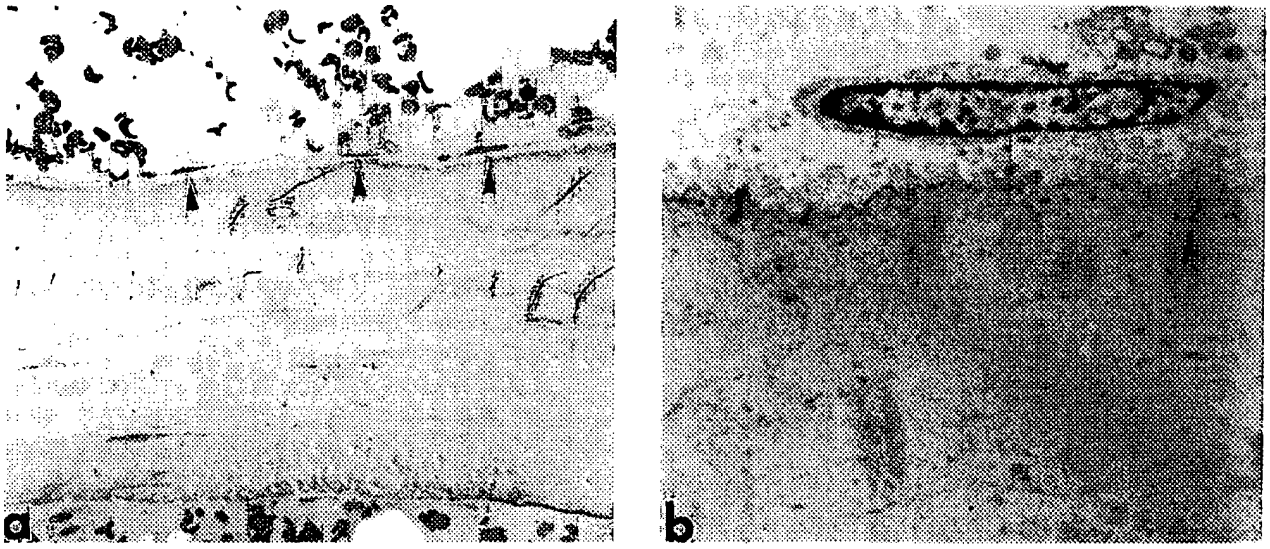

Figure 7. The iliac bone of the control (OA) patient. (a) A semithin section. The surface of the bone trabecula is apparently smooth with frequent lining cells (arrowheads). A moderate number of erythroid cells are present surrounding the trabecula (×400). (b) Electron micrograph of the iliac bone trabecula. The typical normal bone trabecula as shown here is characterized by the clear margin (lamina limitans) and a lining cell closely adhering to the surface (×7200).

ic cells in RA bone marrow have been considered immature cells of myeloid lineage, as noted in this study (Figure 9A): myeloperoxidase positive granules were observed in the cytoplasm of these granulocytes adjacent to irregularly contoured trabeculae. Some myeloperoxidase positive granules were also found dispersed along the trabecular margin (Figure 10).

Electron microscopic observation of iliac bones. Ultrastructural examination of iliac bones revealed no destructive changes in OA control patients (Figure 7B). In less severe RA, although mononuclear myeloid cells were present close to the surface of the trabecula, no apparent alterations were detected. On the surface of the trabecula of less severe RA, even a lining cell was clearly observed (Figures 8A, 8B). On the other hand, in severe RA, a number of immature myeloid cells surrounded bone trabeculae with irregular margins (Figures 9A, 10, 11A); lining cells were not observed (Figures 9B, 10.

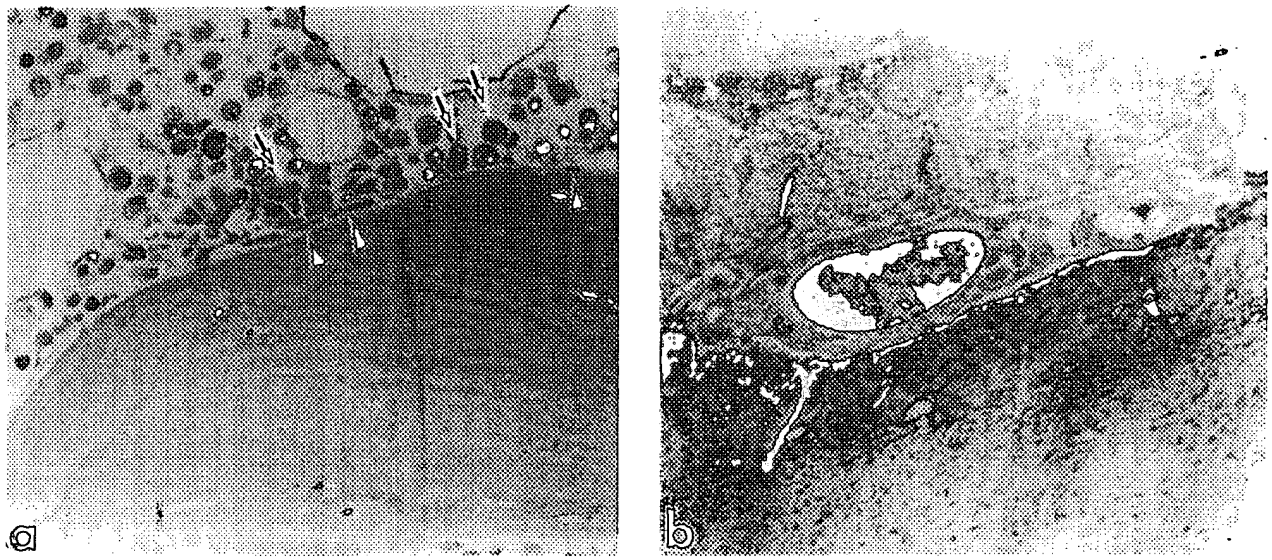

Figure 8. Iliac bone of a patient with less severe RA. (a) A semithin section. A number of mononuclear hemopoietic cells (arrows) are present on the relatively smooth surface of the bone trabecula. Bone lining cells are clearly visible (arrowheads) on the surface of the trabecula (×400). (b) An electron micrograph of the iliac bone trabecula. No destructive changes could be observed even at this magnification; a long, flat lining cell is visible on the surface of the trabecula, and the lamina limitans is readily identifiable (×7200).

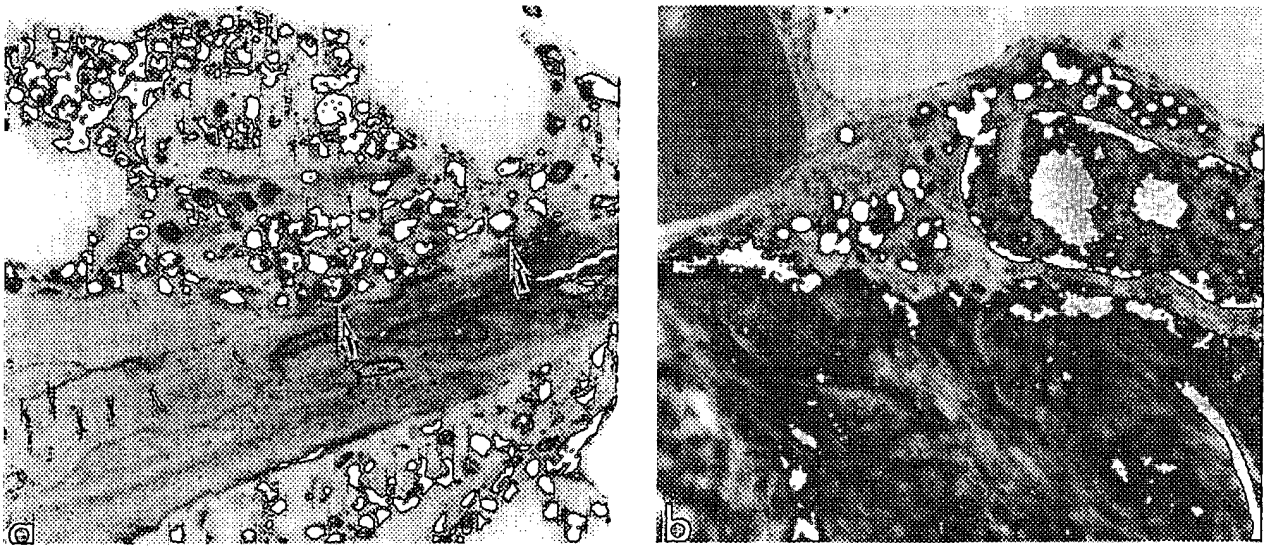

Figure 9. Iliac bone of a patient with severe RA. (a) A semithin section. Surface of the bone trabecula shows a ragged contour. A number of mononuclear cells (arrows) are attached to the ragged surface (×400). (b) Electron micrograph of iliac bone trabecula. A granule-containing mononuclear immature neutrophil (promyelocyte–myelocyte) is shown typically lying on and adhering directly to the surface of the trabecula with no intervening lining cells (×7200).

11A); myeloid cells closely encompassing the trabeculae were seen at the promyelocyte–myelocyte stage (Figures 9B, 11A). In addition to ragged margins frequently observed at the light microscopic level (Figures 9A, 10), obviously destructive alterations of the trabeculae could be observed in severe RA specimens (Figures 11A, 11B), but not in the less severe RA.

Some of the cells of myeloid lineage adhering to trabeculae were in a degeneration process (Figure 11B); no cell boundary could be discerned; close to the trabecula, small electron dense granules, apparently similar to those of neutrophils, were scattered extracellularly; in addition to granules, even mitochondria could be observed extracellularly. The collagenous fibers in some trabeculae became obscure, in particular in places where promyelocytes–myelocytes were clearly visible in the close vicinity of the trabecula; disappearance of the collagenous fibers was more obvious when

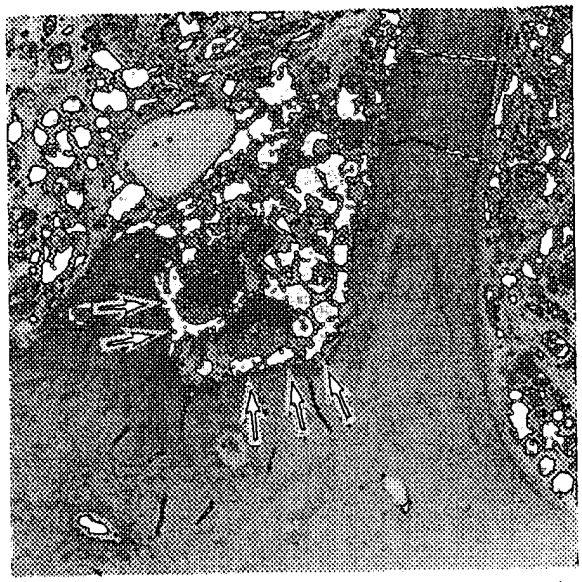

Figure 10. A semithin section of the iliac bone of the patient with RA stained for myeloperoxidase. Myeloperoxidase positive granules are abundant along the irregular margin of the trabecula (arrows), as well as in some mononuclear cells (arrowheads) (a, b ×440).

granules were dispersed even in the bone matrix or in the matrix surrounding the trabecula (Figures 11C, 12A, 12B). Indeed, abundant electron dense granules were observed around the fragmented bone trabeculae, and ultimately these scattered granules were myeloperoxidase positive (Figures 12A, 12B).

DISCUSSION

As in previous studies[7,8], enhanced granulopoiesis in RA bone marrow was evident, characterized by high cellularity and by a large fraction of immature myeloid lineage cells (CD15+CD16–) with a reciprocal relative decrease of mature cells (CD15+CD16+). Enhanced granulopoiesis was found in the iliac bone marrow, remote from the affected articular bones and cartilages, strongly implying that this change in hematopoiesis is a generalized form, not a local symptom. Immature myeloid lineage cells (promyelocytes–myelocytes) were also found even in PB.

Bone trabeculae with ragged margins, frequently observed in severe RA specimens in this study, were closely surrounded by immature myeloid cells. Some of these immature cells

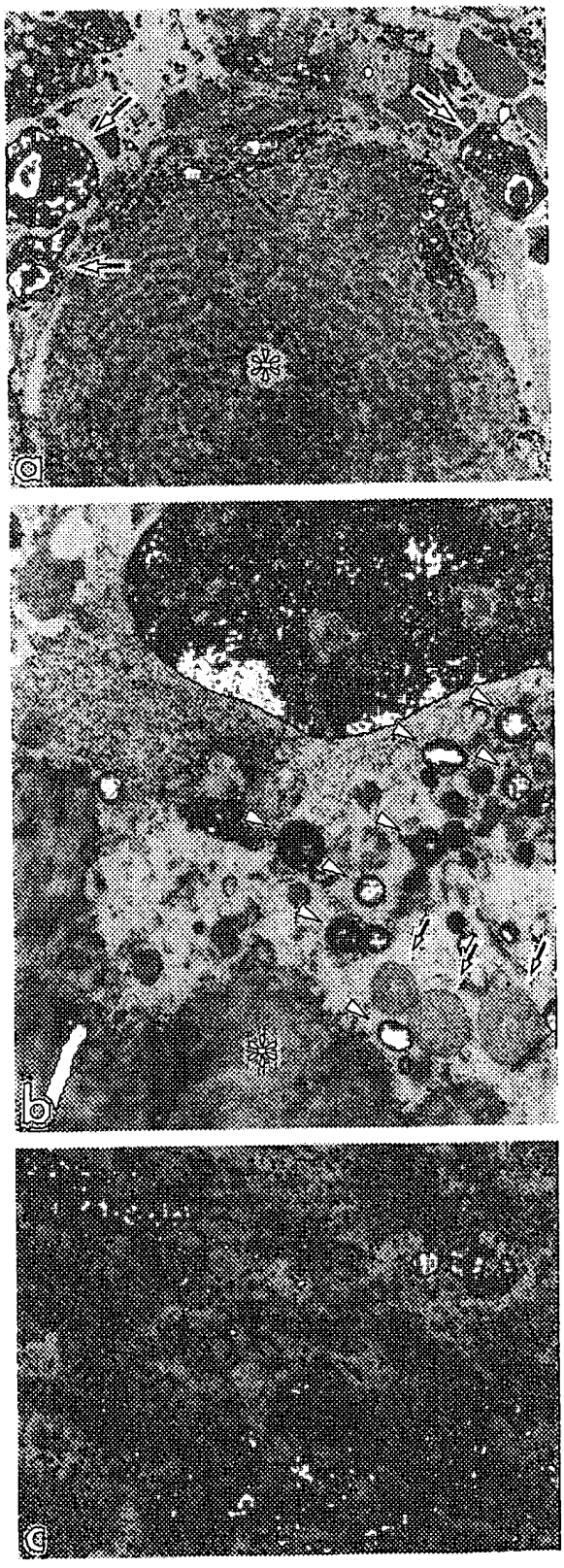

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Figure 11. Electron micrographs of the iliac bone trabecula of a patient with RA. (a) Myeloid cells (arrows) are localized on the surface of bone trabecula (*). Electron dense granules are scattered around the bone trabecula. (b) The cell appears to have lost the cytoplasmic membrane; mitochondria (arrows) and many electron dense granules (arrowheads) are dispersed extracellularly, and are almost adhering to the bone matrix (*). (c) Higher magnification of part of the bone matrix of (a). Typical collagenous fibers with periodic striations are no longer visible (a ×3670; b ×13,000; c ×24,500).

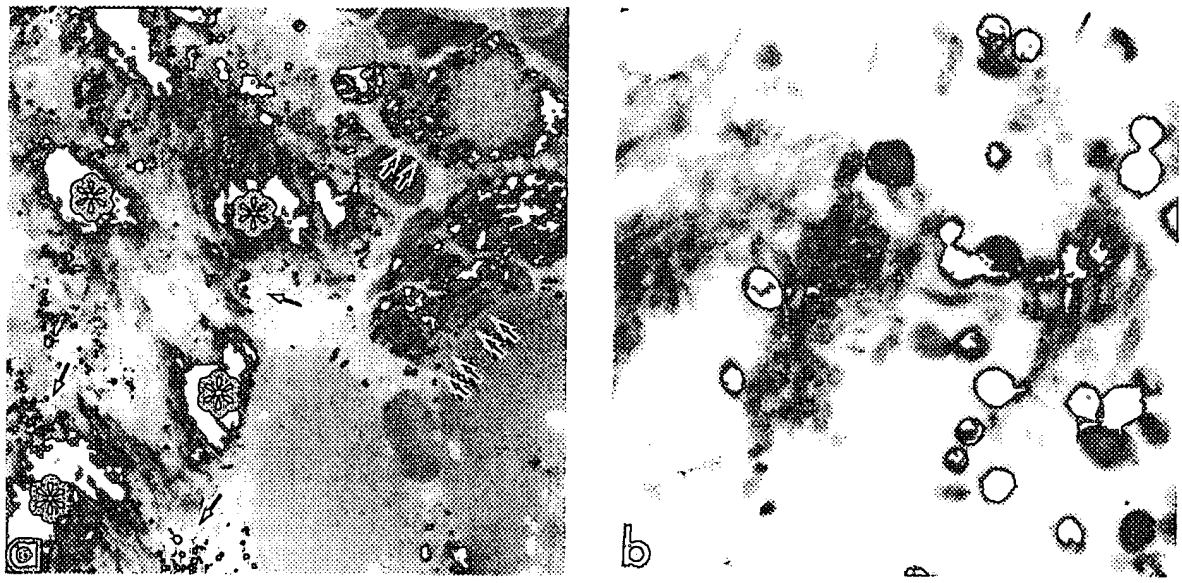

Figure 12. Electron micrographs of the iliac bone of a patient with RA stained for myeloperoxidase. (a) Electron dense (myeloperoxidase positive) granules (arrows) are visible in areas surrounding the fragmented trabecula (*) and within mononuclear cells adhering to the trabecula (double arrows). (b) Unraveled collagenous fibrils with periodicity and fibrils without periodicity, together with myeloperoxidase positive granules (a ×2000; b ×16,800).

were found ruptured, and intact organelles like mitochondria as well as granules considered to have been released from ruptured cells were scattered around the irregularly shaped trabeculae.

Scattered granules in the extracellular matrix at the margin of and even inside the trabeculae were persistently observed. These granules were similar in size and morphology to granules present in immature myeloid cells, and thus it was suspected that these granules were released from the neutrophils frequently detected on the surface of trabeculae.

Further, histochemical techniques for electron microscopic detection of myeloperoxidase clearly showed that both immature neutrophilic granules and those scattered extracellularly were positive for the enzyme. This finding suggests that, even in immature neutrophils, enzymes in cytoplasmic granules were active, confirming the functional significance of immature neutrophils in terms of enzymatic activity, and that granules scattered extracellularly and inside the trabeculae were most likely to be of immature neutrophilic origin.

Collagenous fibers were often found in the degeneration process in irregularly shaped trabeculae; fibers were severed into small fragments, and the periodicity characteristic for collagenous fibers frequently disappeared from fragmented fibers. Around these trabeculae, immature myeloid cells were frequently observed; granules were also scattered around and even inside these trabeculae.

From these findings and observations, a hypothesis could be deduced: in some patients with severe RA, granulopoiesis is extensively enhanced due to persistent articular inflammation; overproduced neutrophils begin accumulating and adhering to bone trabeculae in the bone marrow; for an unknown reason, some of them rupture and eventually release cytoplasmic granules extracellularly toward the bone trabeculae. In turn, destructive enzymes would be activated, and collagenous fibers gradually degraded; finally, the bone matrix might be broken down and margins of trabeculae would become irregular. Whether mature neutrophils were similarly involved in bone degradation could not be determined.

Although adrenocortical steroid has been reported to enhance the egress of granulocytes from bone marrow[16], there was no significant difference in the numbers of the immature and mature granulocytes in comparisons of bone marrow between steroid treated patients with RA and those not treated with steroid (data not shown; Ohtsu, *et al*, unpublished observations). We considered that the steroid had no effect on numbers of granulocytes in this analysis.

To investigate other possible causes for the elevated granulopoiesis in RA bone marrow, the level of granulocyte-colony stimulating factor in the bone marrow plasma was measured, but an increased level was not consistently detected in patients with RA (data not shown). At present, the cause for the enhanced granulopoiesis in RA bone marrow remains to be elucidated.

In contrast to the accumulation of immature myeloid cells, the relative fraction of mature CD15+CD16+ myeloid cells decreased, although their absolute number remained at the normal level. Differentiation might have been arrested, although incompletely, at some time between the myelocyte and metamyelocyte stages. Another striking feature is the presence of promyelocytes–myelocytes in PB. Except for

leukemic conditions, it is extremely rare to find these immature myeloid cells in the periphery. In the bone marrow of patients with severe RA, lining cells were often absent around irregular shaped trabeculae, suggesting that in severe RA bone marrow, not only bone trabeculae, but some stromal meshwork holding hematopoietic compartment might be destroyed.

Ochi, *et al*[6-8] reported abnormal myeloid lineage cells bearing an oncofetal marker in RA bone marrow, but so far their functions have not been delineated. Our results strongly suggest that overproduced immature neutrophils might be involved in generalized bone destruction. This type of bone degradation is quite different from conventional bone absorption by osteoclasts[17-20]. More direct evidence would be necessary to clearly confirm a distinctive role of neutrophils in a novel type of bone destruction.

Neutrophilic granules contain many destructive enzymes, such as matrix metalloproteinases (MMP), elastases, and peroxidases[21-25]. Okada, *et al* demonstrated that MMP-9 could degrade collagenous fibrils of demineralized bone fragments *in vitro*[26], strongly indicating that proteolytic enzymes present inside the granules could digest bone organic matrix.

The morphological data presented in this study indicate that the neutrophil and its granules might be responsible for destruction of the bone and collagenous fibers, the phenomenon clearly observed in RA bone specimens in areas remote from the inflamed joints. A report by Gillespie, *et al*[27] that PMN were able to degrade bone in culture in osteomyelitis would further support this hypothesis.

Generalized osteoporosis in RA has been considered to be due to enhanced bone resorption by osteoclasts, defective bone formation, or defective production of cytokines[28,29]. Abundant macrophages and osteoclasts were found in bones of the affected joints; bone erosion in the inflamed regions seemed to be caused by these cells[30,31]. Although involvement of osteoclasts in generalized bone destruction in severe RA may play a role, the morphological evidence we have presented strongly suggests a novel mechanism for bone destruction by neutrophils; in the hyperplastic bone marrow of patients with severe RA, immature neutrophils may cause generalized bone destruction.

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